Temperature-Mediated Alterations of the Plant Apoplast as a Mechanism of Intracellular Freezing Stress Avoidance

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ABSTRACT

Cold hardy Japanese bunching onion (*Allium fistulosum* L.) was used as a novel model system to examine the role of the apoplast in intracellular freezing avoidance. Since intracellular freezing avoidance is critical to both sensitive and resistant plants, non-acclimated and cold acclimated onion tissue was compared. The large 250 (length) x 50 (width) x 90 μm (thickness) intact single cell epidermal layer system allowed direct observation of the freezing process during freezing and thawing on a single cell basis in live intact tissues in non-acclimated (23/18°C) and acclimated (2 weeks at 12/4°C) plants. Under acclimation treatment, freezing resistance increased from an LT_{50} of -12°C at zero time to -27°C after two weeks exposure. Ice nucleation was always initiated in the apoplastic space in both non-acclimated and acclimated cells and then penetrated into the intracellular space. We provide direct evidence that lethal intracellular freezing damage was avoided in cold acclimated and $CaCl_2$ treated cells through visible blockage of ice propagation from the apoplastic space to the intracellular region. Subsequent investigation of the apoplastic space revealed cold acclimation increased: a) the insoluble crude cell wall and pectin (galacturonic acid) content, b) total cell wall protein quantity, c) pectin methylesterase activity in the apoplastic space particularly in epidermal cells compared to non-epidermal cells, and d) the levels of un-methylated pectin. The increasing freezing resistance of Japanese bunching onion more depends on the efficiency of PME not only the increase in the amount of apoplast compositions. Collectively, these changes likely led to the observed significant reduction in cell wall permeability. In this way, freezing stress resistance may potentially be increased through avoidance of intracellular freezing by altering key factors related to apoplast permeability and blocking ice propagation across the cell wall.

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1.0 INTRODUCTION

Temperature has had a primary influence on adaptation throughout plant evolutionary history. Future temperatures are predicted to increase under global climate change with night temperatures increasing more than day temperatures (Easterling and Horton, 1997; IPCC, 2007). According to Gu *et al*. (2008) and as reviewed in Storey and Tanino (2012), injury due to low temperature stress will become even more important under global warming, not less. Since temperature also influences the ability to increase freezing resistance through the process of cold acclimation, the ability to acclimate in response to temperature is an important survival mechanism in temperate plants.

Kozlowski and Pallardy (1997) divided cold acclimation into two sequential stages: Stage I: The accumulation of carbohydrates and lipids in the autumn, which are substrates and energy sources for the next metabolic phases; Stage II: Freezing temperatures to synthesize proteins and membrane lipids and change structures to acclimate against cold winters. Cold acclimation is regulated by both low temperature and photoperiod and shares similarities with the important environmental induction parameters of growth cessation and dormancy (Weiser, 1970).

Water diffusivity (Zeuthen, 2001; Welling and Palva, 2006; Yooyongwech, 2008; Kalcsits *et al*., 2009) and carbohydrate accumulation in the cell wall (Olien and Clark, 1993; Livingston *et al*., 2009; Tanino *et al*., 1990, 1991, 2010) are significantly connected with the development of low temperature stress resistance. The regulation of water flow across the cell wall is largely attributed to aquaporins (Carvajal *et al*., 1998) and plasmodesmata (Kuo *et al*., 1974; Anisimov and Egorov, 2002; Liesche *et al*., 2011). Aquaporin genes are downregulated in the transition periods during dormancy phases (Mazzitelli *et al*., 2007). Most of the environmental stress factors, including low temperature, can stimulate the generation of intracellular signals and depositions of callose at plasmodesmata during dormancy (Sivaguru *et al*., 2000; Rinne *et al*., 2011; van der Schoot *et al*., 2011), thus reducing water flow into the cells. However, the role of the apoplastic matrix itself (cell wall and middle lamella) during cold stress has not received much attention**.**

The apoplast is a physical barrier against environmental intrusions, including pathogens and abiotic factors, *i.e.* ice propagation and dehydration stress through excessive water loss.

Plant species in the temperate zones are vulnerable to natural low temperatures and cold hardy plants first freeze outside the plasma membrane in the extracellular space of the apoplast (cell wall, middle lamella) (Levitt, 1980). Water inside the cells will then migrate out to freeze in the apoplast, following its vapour pressure deficit, thus leading to cellular dehydration and osmotic shrinkage of the cell (Dowgert and Steponkus, 1984; Ruelland *et al*., 2009). To a certain degree, the mechanical characteristics of cell walls appear to adjust to the kinetics and the range of dehydration being stimulated by freezing, thereby improving the ability of plants to resist extracellular freezing (Solecka *et al*., 2008). The freezing process can result in cell dehydration stress. As the temperature is lowered, the more serious cell dehydration becomes (Pearce and Ashworth, 1992). However, dehydration resulting from extracellular freezing can help cells to avoid intracellular freezing and increase survival at very low, even liquid-nitrogen temperatures (Sakai and Larcher, 1987). Conversely, if intracellular freezing occurs, the cells will die (Levitt, 1980). Thus, the apoplast matrix represents a common barrier to both water loss out from the cell (freezing tolerance), as well as a barrier to ice penetrating into the intracellular space (freezing avoidance).

Gusta *et al*. (2009) indicated there are seven different freezing types in plants, such as partially or fully cold acclimation, and supercooling or deep supercooling. However, regardless of the freezing process, the common critical factor to frost survival is avoidance of intracellular freezing. Since the apoplast represents the first barrier to ice, the changes within the apoplast under cold acclimation will be examined in this thesis. While several studies have examined modification of ice crystals within the apoplastic space by carbohydrates (Griffith *et al*., 1985; Olien and Lester, 1985; Olien and Clark, 1993), this thesis focuses on the apoplast as a barrier preventing ice propagation across the cell wall to freeze the intracellular space.

The principal elements in the apoplast (consisting of middle lamella and cell wall) are polysaccharides consisting of monosaccharides. The cell wall fraction is a biphasic structure consisting of cellulose microfibrils held together by a gel-like matrix which constitutes about two-thirds of the cell wall's dry weight and is composed of cross-linking extensin glycoproteins and non-cellulosic polysaccharides including pectins and hemicelluloses (Fry, 1986; Pelloux *et al*., 2007). Other apoplastic components are lignin, suberin, wax, cutin and water. The middle lamella is primarily composed of pectins. Pectins are chiefly divided into xylogalacturonan, homogalacturons (HGA), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). These

components serve many functions including determining apoplast porosity, providing a charged wall surface supporting cell-cell adhesion (i.e., middle lamella), cell-cell identification, pathogen recognition and other roles.

The gelation and rigidity of the cell wall is regulated by pectin substances through pectin methylesterase (PME) (Willats *et al*., 2001). PME catalyzes the de-esterification of pectin methyl groups, permitting Ca^{2+} binding to the carboxylate ions and generating calcium cross-linkages (Pelloux *et al*., 2007). In poplar cambium, acidic pectins and calcium causes early stiffening of maturing phloem cells (Guglielmino *et al*., 1997a) and apparent changes in PME isoforms (Guglielmino *et al*., 1997b). Cell wall-bound PME's are temperature- sensitive (Anthon and Barrett, 2006) but most of this evidence comes from the food processing literature and few such studies have been done on living plants. Wisniewski and Davis (1995) illustrated deep supercooling in trees is associated with pectins preventing water loss and ice nucleation within the cells. A subsequent study (Solecka *et al*., 2008) demonstrated cold acclimation of winter oil-seed rape is increased by the temperature-dependent PME. Although it is well-known that the mediation of water movement is critical to plant growth and development, there are few studies examining the role of the cell wall, and pectins in particular, in regulating water flow and ice propagation (Wisniewski and Davis, 2005). Most studies of the apoplast water migration have been focused on aquaporins (Mazzitelli *et al*., 2007) and plasmodesmata (Liesche *et al*., 2011).

Previous studies have shown that Japanese bunching onion is a suitable system for studying changes in the structure and components of the cell wall in response to intracellular freezing avoidance (Tanino *et al*., 2013). Japanese bunching onion is a very hardy species and can tolerate extreme environmental stress. The advantage of this plant for apoplast studies is an easy to peel intact single epidermal layer devoid of chlorophyll that facilitates immunofluorescence studies. In addition, the cells are large (250 x 50 x 90μm). The plant acclimates quickly to cold (increases in freezing resistance from -12^{o}C to -27^{o}C in two weeks of acclimation). All of these advantages make Japanese bunching onion an ideal system to study freezing stress mechanisms.

1.1 Hypothesis

Reduced permeability of the apoplast through pectin modification will increase freezing avoidance and freezing stress survival.

1.2 Objectives

1) To determine if the permeability of the apoplast (cell wall/middle lamella) plays a role in freezing avoidance.
2) To determine if cold acclimation decreases apoplastic permeability through apoplast compositional changes.

2.0 LITERATURE REVIEW

2.1 Freezing Stress

Plant stress can be divided into two types: biotic stress and abiotic stress. Biotic stress involves plant damage from other living organisms, such as bacteria, fungi, and insects. Abiotic stress, also known as environmental stress, is referred to as the adverse influences on the existing organisms resulting from non-living factors (Ortbauer, 2013). The non-living factors include: extreme temperatures, drought, flood, salt, wind, etc. Each year, millions of tonnes of potential crops and other plants yield fail to materialize because of various abiotic stress factors that impact the growth and maturation of the plants (Xiong and Zhu, 2001; Kozlowski, 2002). Plants are commonly very susceptible to environmental stimuli. At the same time, plants can physiologically and structurally acclimate and adjust in order to survive in their environment.

Based on the research of Intergovernmental Panel on Climate Change (IPCC, 2014), the extreme temperatures have been changing for decades. It is predicted that the global average surface temperature may rise from 2.5 to 7.8ºC in 2100 (IPCC, 2014). Increasing temperature has a negative effect on agriculture because of the frequency of drought, pests and diseases which may arise. There will be fewer cold days and cold nights and frosts, more hot days and hot nights and heat waves. In addition, snow and ice covers are decreasing widely. Snow and ice plays a vital role in the global climate system and normally provides the avoidance protection for plants. The growing season will also be extended in which spring occurs earlier and autumn later. This has already increased damage due to early spring bloom which is more susceptible to frost damage. In 2007, over $2 billion US damage occurred to frost sensitive horticulture crops (e.g. bedding plants, blossoms of pear, apple and peach) in the USA over a two day weekend (Gu *et al*., 2008). Sensitive plants only have one mechanism of freezing stress resistance and that is frost avoidance. Therefore, it will become increasingly critical to understand the mechanism of frost avoidance in plants.

2.1.1 Freezing Process

One of the most significant stresses is low temperature which includes two types of cold stress: chilling stress and freezing stress. Chilling stress is commonly defined to occur at low temperatures but above zero (from 0 to 10^{o}C) without ice formation, while freezing stress is

exerted at negative temperatures with ice nucleation in plant tissues (Thomashow, 1999; Kolaksazov *et al*., 2013). Freezing stress can affect the growth of aerial parts of plants (such as leaf expansion), causing wilting, chlorosis and necrosis (Mahajan and Tuteja, 2005; Sanghera *et al*., 2011), alter root growth (Noh *et al*., 2009), and even result in cell collapse and death (Stefanowska *et al*., 1999; Mahajan and Tuteja, 2005).The freezing effect on plant cells is described in Figure 2.1. Freezing injury is thought to occur primarily in the plant plasma membrane mainly due to the severe dehydration induced by freezing (Pearce and Willison, 1985; Steponkus *et al*., 1998; Pearce, 2001; Thomashow, 2001). Once the ice crystals penetrate the symplast or grow directly into the intracellular spaces, it is immediately fatal since the intracellular ice can physically destroy the structures of plasma membrane and the integrity of the living protoplasm and this process is irreversible (Burke *et al.*, 1976; Gusta *et al*., 2004).

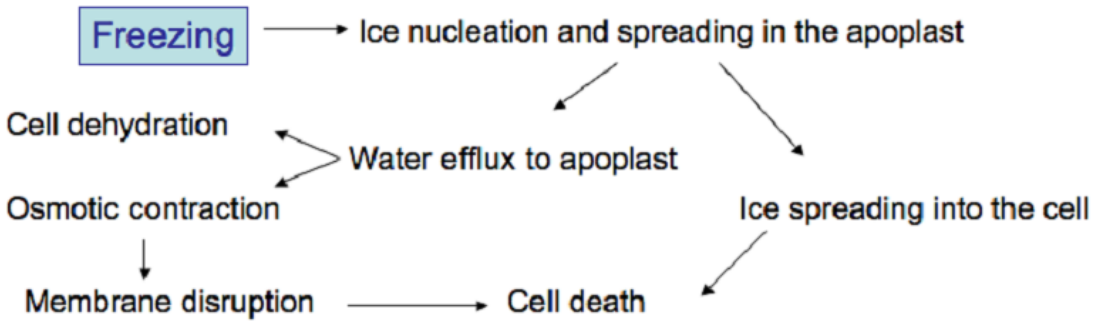


Figure 2.1 The freezing effect on plant cells (Ruelland *et al*., 2009).

Freezing stress in plants can be sorted into at least seven different types (Stushnoff *et al*., 1984; Gusta *et al*., 2009). The first freezing type includes the succulent tender tissues like tomatoes and corn containing high moisture content such as tropical plants. In this pattern, the plants are very sensitive to both chilling and frost and the injury occurs just below freezing from -2°C to -5°C. The second freezing type appears in partially acclimated tissues or mature tissues with its LT_{50} values from -3 °C to -10 °C. The third freezing pattern occurs in fully cold-acclimated hardy species with low water content (< 75% water) such as winter cereals and trees that do not supercool, and for these species the freezing injury is mainly due to extracellular freeze-induced dehydration. The fourth freezing type is related to the ability of certain cells (such as xylem ray parenchyma cells) to supercool. When the cooling rate of the tissue is greater than 3 ºC/hour, the fifth freezing type occurs, which is not regarded as true supercooling. This type

occurs when the low temperature exotherm (LTE) disappears and the temperature of the LTE does not correspond to the killing temperature in slowly cooled seeds. The sixth freezing type is related to differential organ supercooling and extraorgan freezing in non-acclimated cereals in which contains barriers to prevent ice penetration. The seventh freezing type involves vitrification or glass formation which occurs at sub-zero temperatures when water is converted into a glass-like amorphous solid but does not form the crystalline structure.

At the cellular level, ice crystals in freezing resistant plants are first generated on the surface of cell walls and spread throughout the extracellular space (Burke *et al*., 1976; Guy, 1990). Ice can be limited to the external regions of the cell by the intact cell wall and plasma membrane and a slow cooling rate. This process is called extracellular freezing (Levitt, 1980; Guy, 1990). The intracellular water diffuses down its vapour pressure gradient caused by extracellular freezing, to form ice crystals in the apoplastic space resulting in cell dehydration and cell osmolarity, induces cell shrinkage and finally cell collapse (Ashworth *et al*., 1988; Yelenosky and Guy, 1989; Guy, 2003). Accompanying the falling temperatures, more intracellular water diffuses out and the extent of dehydration increases (Johansson and Krull, 1970; Gusta *et al*., 1975; Ashworth and Pearce, 2002). Once the dehydration exceeds the critical range, the cells are killed by a combination of low temperature and desiccation stress.

The other freezing condition is termed intracellular freezing which may occur due to ice nucleation throughout the protoplast and vacuole (Levitt and Scarth, 1936) or the penetration to the intracellular spaces via the extracellular ice (Mazur, 1969; Steponkus *et al*., 1983) when the cooling rate is rapid ($\geq 3^{o}$C/hour). The accumulation of ice in the middle lamella can potentially lead to cell disruption partly due to the ice adhesions between the cells, cell walls and membranes (Levitt, 1980). The direct consequence of intracellular freezing is lethal to all the cells, which may be related to the physical destruction of the plasma membrane due to internal ice crystal propagation (Muldrew *et al*., 2004). However, even though the cooling rate is slow ($< 1^{o}$C/hour), there is an exception—intracellular freezing may not take place but deep supercooling occurs in some tissues such as xylem (Wisniewski and Davis, 1995), bark (Quamme, 1985) and flower buds (Sakai, 1978; Ashworth, 1984). Supercooling is the process where a liquid or a gas does not become a solid state below its freezing point when lowering the temperature (Moore and Molinero, 2011).

The freezing process in plants can be driven by: water potential in both extracellular and intracellular water (Wenkert, 1980; Levitt, 1972, 1980), all intact effective cell wall and plasma membrane serving as a freezing barrier to avoid intracellular freezing (Dowgert and Steponkus, 1984; Steponkus *et al*., 1983; Levitt and Scarth, 1936; Steponkus *et al*., 1982), the existence of heterogeneous nucleators (Burke *et al*., 1976; Sakai and Larcher, 1987), and the rate of cooling (Mazur, 1963, 1969; Steponkus *et al*., 1983; Guy, 1990). Ice formation in plant tissues is triggered in nucleation sites where water molecules come together to form an ice nucleus when exposed to low temperature (Burke and Lindow, 1990). This process belongs to heterogeneous nucleation which can be induced by other substances including ice nucleation-active (INA) bacteria, the biological molecules and structures, organic and inorganic debris (Pearce, 2001). The nucleation sites are usually of a specific size and shape, and are related to the composition of the cell wall. Both composition and structure of the cell wall can assist the plant tissues to control the ice nucleation sites. Ice formation is thought to initially occur in these areas with the least negative osmotic potential such as the large vessels of xylem of leaves and stems (Levitt, 1980; Sakai and Larcher, 1987), thereafter spread rapidly into the extracellular spaces of other tissues. Recent evidence indicates ice nucleation can initiate in the phloem and cortical tissue of *Rhododendron japonicum* and *Cornus officinalis* (Ishikawa and Sakai, 1982), stems of blueberry (Kishioto *et al*., 2014) and *Rhododendron japonicum* flower bud scales (Ishikawa *et al*., 2015).

Due to their immobility, plants have to sense and respond to the fluctuating environmental temperature. One of the amazing abilities of plants is to increase their freezing resistance through pre-exposure to low but non-freezing temperatures, which is called cold acclimation (Levitt, 1980; Sakai and Larcher, 1987; Guy, 1990; Steponkus, 1990; Thomashow, 1999). Numerous alterations in morphology, metabolism, physiology, and gene expression occur during cold acclimation (Thomashow, 1999; Levitt, 1980; Xin and Browse, 1998) in order to increase the possibility of plant survival under low temperature and/or freeze-induced dehydration stress. With these alterations, plants can tolerate the extracellular dehydration stress induced by extracellular freezing (freezing tolerance) and/or avoid intracellular ice nucleation (freezing avoidance). The multiple mechanisms of plant resistance usually do not exist independently but occur in the same organs to defense freezing injury. For example, the flower buds of *Rhododendron japonicum* avoid freezing damage by supercooling (freezing avoidance) (Ishikawa and Sakai, 1981) while the flower buds of *Rhododendron japonicum* can tolerate the

accumulation of ice in a particular space outside the organ such as bud scale which can be regarded as a barrier through partial dehydration (freezing tolerance) (Ishikawa and Sakai, 1982). In addition, the existence of barriers such as the cell wall and plasma membrane can help to isolate the cells from extracellular freezing and inhibit the loss of water molecules to the surrounding ice.

2.1.2 Freezing Avoidance and Freezing Tolerance

There are two major categories of freezing resistance: freezing avoidance and freezing tolerance (Levitt, 1972, 1980). Freezing avoidance can occur when plants affect the freezing process by preventing the penetration of ice crystals into the tissue or the cellular levels. Examples include deep supercooling of internal tissue water which helps to avoid cell dehydration caused by extracellular ice formation (Ishikawa and Sakai, 1981; Wisniewski *et al*., 1987) and extraorgan freezing (Ishikawa and Sakai, 1982; Fujikawa *et al*., 2000, 2009; Kuroda *et al*., 2003; Endoh *et al*., 2009). In addition, freezing avoidance can also include shedding or insulating vulnerable plant parts (Raulston and Tripp, 1994). For example, alpine plants warm their shoots by burying themselves below ground and thereby avoiding severe freezing stress, while permitting leaves to freeze independently in the air (Körner, 2003). In this way, separation of ice nucleation and the prevention of ice propagation from the frozen shoot enable susceptible reproductive structures to avoid freezing stress (Hacker *et al*., 2011).

Freezing avoidance regulates the nucleating position of ice crystals in the plant (Wisniewski *et al*., 2014). Heterogeneous ice nucleation, triggered by ice nucleation active substance (INAS) including inorganic substances such as silver iodide (Vonnegut, 1947) and organic substances such as ice-nucleating-active bacteria (e.g. *Pseudomonas syringae*) (Ashworth and Kieft, 1995) is different from homogeneous ice nucleation, in which ice form autonomously (Wisniewski *et al*., 2014). Silver iodide (AgI) is an effective nucleus agent to form the ice crystals since the crystal structure (the dimensions of the unit) of AgI is similar to ice (Vonnegut, 1947). Therefore, the application of AgI is easy to initiate ice nucleation in cellular space. Occurrence of extrinsic or intrinsic ice nucleation depends on the presence of INAS on the plant surface or within the plant (Pearce, 2001). However, INAS are not the exclusive trigger for extrinsic ice nucleation. Wettability of plants plays an important role too. Reducing leaf wettability leads to the reduction of extrinsic ice nucleation in potatoes, grapevine, lemon and tomato (Wisniewski *et al.*, 2002). Intrinsic ice nucleation is regulated by both INAS and anti-ice

nucleating substances, such as antifreeze proteins (AFPs) and fructans. While AFPs are regarded to play a crucial role in affecting the shape and size of ice crystals (Griffith *et al*., 2005), fructans are thought to stabilize cell membranes and change the morphology and growth pattern of ice crystals (Olien and Marchetti, 1976; Valluru and Van den Ende, 2008). As one of the most well-known examples of freezing avoidance is supercooling, this phenomenon allows water in plant cells to remain in a liquid phase and avoid freezing at low, subfreezing temperatures (Wisniewski *et al*., 2014) due to solutes within the cell solution reducing the freezing point. In addition, anti-nucleating substances such as tannins and related polyphenols and glycosides can also enhance supercooling extent (Kuwabara *et al.*, 2013; Kasuga *et al*., 2010). Due to deep supercooling, apoplastic water freezing of xylem ray parenchyma cells can be avoided (George *et al*., 1982). The intracellular space does not lose water even in the presence of ice in apoplastic spaces via deep supercooling (Fujikawa *et al*., 1994), thereby maintaining the original volumes. Extraorgan freezing is freezing of large extracellular cavities such as bud scales which serve as preferred freezing sites in tree buds (Sakai and Larcher, 1987). Ice propagation is prevented in the dry region created by water withdrawal to the extracellular cavities.

Once intracellular freezing occurs, it is lethal for all biological materials (Levitt, 1980). Therefore, different types of barriers exist in plants, isolating the supercooled cells from external nucleation in order to block the ice propagation and prevent water loss to surrounding ice. Thermal ice barriers refer to plant structures that are thermally buffered to separate them from those directly exposed to freezing stress. Structural ice barriers are associated with compositional alteration of the plasma membrane and cell wall architecture to inhibit ice propagation (Ashworth and Abeles, 1984; Steponkus, 1984; Uemura and Steponkus, 1997). For instance, a break in the epicuticular layer provides a pathway for ice to physically propagate into the leaf. Hence, thick cuticles serve as effective structural ice barriers. Supercooled cell walls are generally thicker and less flexible (Fujikawa *et al*., 1999). The xylem ray parenchyma cells including pit membrane in woody plants (Wisniewski and Davis, 1989; Wisniewski *et al*., 1991b), and flower buds in many *Prunus* species and other plants (Quamme, 1974; Graham and Mullin, 1976; Biermann *et al*., 1979; Wolpert and Howell, 1984; Sakai *et al*., 1986) are also regarded as ice barriers resulting from physical characteristics to prevent ice propagation and water loss (Chalker-Scott, 1992). Through use of Infrared Video Thermography (IRVT) it is known that ice in cranberry stems cannot propagate into the fruit (Workmaster *et al*., 1999). Ice

barriers are also present in the crowns (Single and Marcellos, 1981) and at nodes (Zámećník *et al*., 1994; Wisniewski *et al*., 1997; Pearce and Fuller, 2001) of cereals and beans, respectively, where they delay the spread of ice throughout the tissue.

Freezing tolerance is defined as the allowance of ice nucleation but the strain is decreased or eliminated through resistance to extracellular freezing dehydration and cell shrinkage. Thus, freezing tolerance refers to the capability of plants to tolerate the presence of ice in their extracellular tissues and the concomitant dehydration stress through cold acclimation (Gusta and Wisniewski, 2013). The tolerance generally associated with biochemical adaptations and alterations in gene regulation. Freezing injury in plants is partly due to interruption of plant water status when cellular dehydration appears because of ice generation outside the cell in the freezing process (Ashworth and Kieft, 1995; Verslues *et al*., 2006). Freezing resistance mechanisms could be referred to as dehydration resistance mechanisms which provide protection to cellular architecture from the impacts of dehydration. There are various mechanisms of dehydration or freezing resistance, including: (1) protection and repair mechanisms which are a primary means to tolerate desiccation stress induced by freezing and involve late-embryogenesis-abundant (LEA) (Close, 1997) and dehydrin proteins (COR15), compatible solutes (Hincha and Hagemann, 2004), ROS scavenging (Apel and Hirt, 2004); (2) a collection of solutes and cell wall hardening, which is associated with dehydration avoidance mechanisms (Verslues *et al*., 2006); (3) accumulation of hormones such as ABA, which is a key regulatory factor in reducing dehydration stress via regulating stomatal conductance (Schroeder *et al*., 2001) and accumulating compatible solutes (Ober and Sharp, 1994); (4) the changes of plasma membrane organization and functionality such as lipids (e.g. increase in the proportion of phospholipids) (Uemura *et al*., 2006) and proteins (e.g. dehydrin family proteins) (Kosová *et al*., 2007) is a critical prerequisite in order to survive from dehydration stress.

Expression of several different types of proteins, including dehydrins (Hanin *et al.*, 2011; Hara, 2010), Cold Responsive proteins (Gilmour *et al*., 1992), increase under low temperature condition. Although not conclusive, these proteins are assumed to be associated with freezing tolerance, since they can protect cellular components from denaturation (Burchett *et al*., 2006) by interacting with sugars to inhibit desiccation (Wolkers *et al*., 2001).

Plants do not gain freezing tolerance capability effectively without the accumulation of sugars (Hurry *et al*., 1994; Dahal *et al*., 2012). Huge differences in cold acclimation capability

between spring (e.g. LT50, -9 ^{o}C) and winter cereals (e.g. LT50, -25 ^{o}C) appears to be due to the inability of spring cereals to effectively accumulate sugars and anthocyanins at low non-freezing temperatures, unlike winter cereals (Hu *et al*., 2001; Hüner *et al*., 1993; Dahal *et al*., 2012).

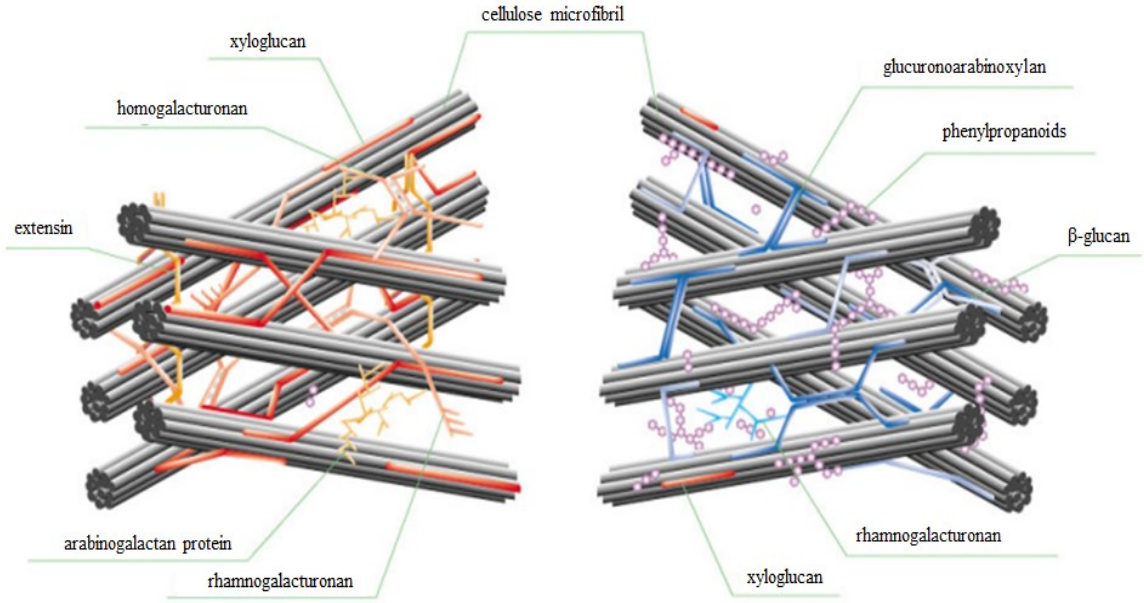
2.2 Apoplast

2.2.1 The Structure of the Apoplast

The apoplast is a free diffusional compartment outside the plasma membrane and includes the walls of living cells and entire nonliving cells, middle lamella, intercellular air spaces, water and solutes (Peterson and Cholewa, 1998; Rose, 2003). The cell wall consists of a complex matrix of polysaccharides and other polymers, and plays a crucial role in plant cell survival. Although appearance and composition of apoplast vary in different cell types (Albersheim *et al*., 2011), basically they are composed of three layers: middle lamella, primary walls and secondary walls (Buchanan *et al*., 2000). Primary cell walls appear in the young cells of growing plant tissues, and mainly consist of cellulose, hemicellulose and pectin (McNeil *et al*., 1984). Secondary cell walls form after the cessation of cell growth; they are highly different in structure and composition compared with primary cell walls, containing cellulose, xylan and lignin. Generally, secondary cell walls are thicker and have more strength than primary cell walls due to the presence of lignin (Vogel, 2008). The middle lamella is a thin layer of material connecting the walls of adjacent cells and is composed almost entirely of pectin. Plant cell walls also contain four types of structural proteins: hydroxyproline-rich glycoproteins (HRGP), arabinogalactan proteins (AGP), glycine-rich proteins (GRPs), and proline-rich proteins (PRPs) (Rose, 2003). In addition, cell walls contain abundant enzymes such as hydrolases and esterases. Cell walls from the epidermis and endodermis may contain higher levels of suberin or cutin than other cells.

The primary cell walls of angiosperms are classified into two distinct types as illustrated in Figure 2.2 (Carpita and Gibeaut, 1993; Yokoyama and Nishitani, 2004; Jones *et al*., 2005; Vogel, 2008). Type I cell walls exist in all dicots and non-graminaceous monocots such as aroids and lilioids and is a network of cellulose microfibrils interlocked with xyloglucan (XyG) and structural proteins and embedded in a matrix of pectic polysaccharides. Type II cell walls are found in the Poaceae and some related monocot families such as rushes and gingers. They consist of similar cellulose microfibril structures as the Type I cell walls but with a framework

interlocked with glucuronoarabinoxylans (GAX). The amount of pectin and structural proteins in the cell wall can be used to distinguish these two types of primary cell walls. Type I cell walls have 20-35% pectin (O'Neil and York, 2003) and 10% structural proteins (O'Neil and York, 2003; Zablackis *et al*., 1995), while Type II cell walls have 5% pectin (Ishii, 1997) and 1% structural proteins (O'Neil and York, 2003). Based on these characteristics, the cell walls of onion (*Allium cepa*) belong to the Type I cell wall (McCann *et al*., 1992; Carpita and Gibeaut, 1993). The onion cell wall contains about 42.4% pectins, 36.6% hemicelluloses and 21% cellulose (Mankarios *et al*. 1980) with 0.7% of xyloglucans and low abundance of phenolics and proteins (McCann *et al*., 1990; McCann *et al*., 1992).



Type I cell wall
(*Arabidopsis thaliana*)

Type II cell wall
(*Oryza sativa*)

Figure 2.2 Schematic structural models of type I and type II walls. Cell walls illustrated are represented by Arabidopsis and rice cell walls, respectively (Yokoyama and Nishitani, 2004).

In Type I cell walls, pectic polysaccharides are one of the key components (Figure 2.3 A), which have a role in determining the pore size of the cell wall (Baron-Epei *et al*., 1988; Carpita and Gibeaut, 1993). The pectins in plant cell walls consist of a complex set of GalA-containing polysaccharides that possess 1,4-linked α-D-galactosyluronic acid (Gal*p*A) residues (Ridley *et al*., 2001; Caffall and Mohnen, 2009). The major types of pectins include homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II), and

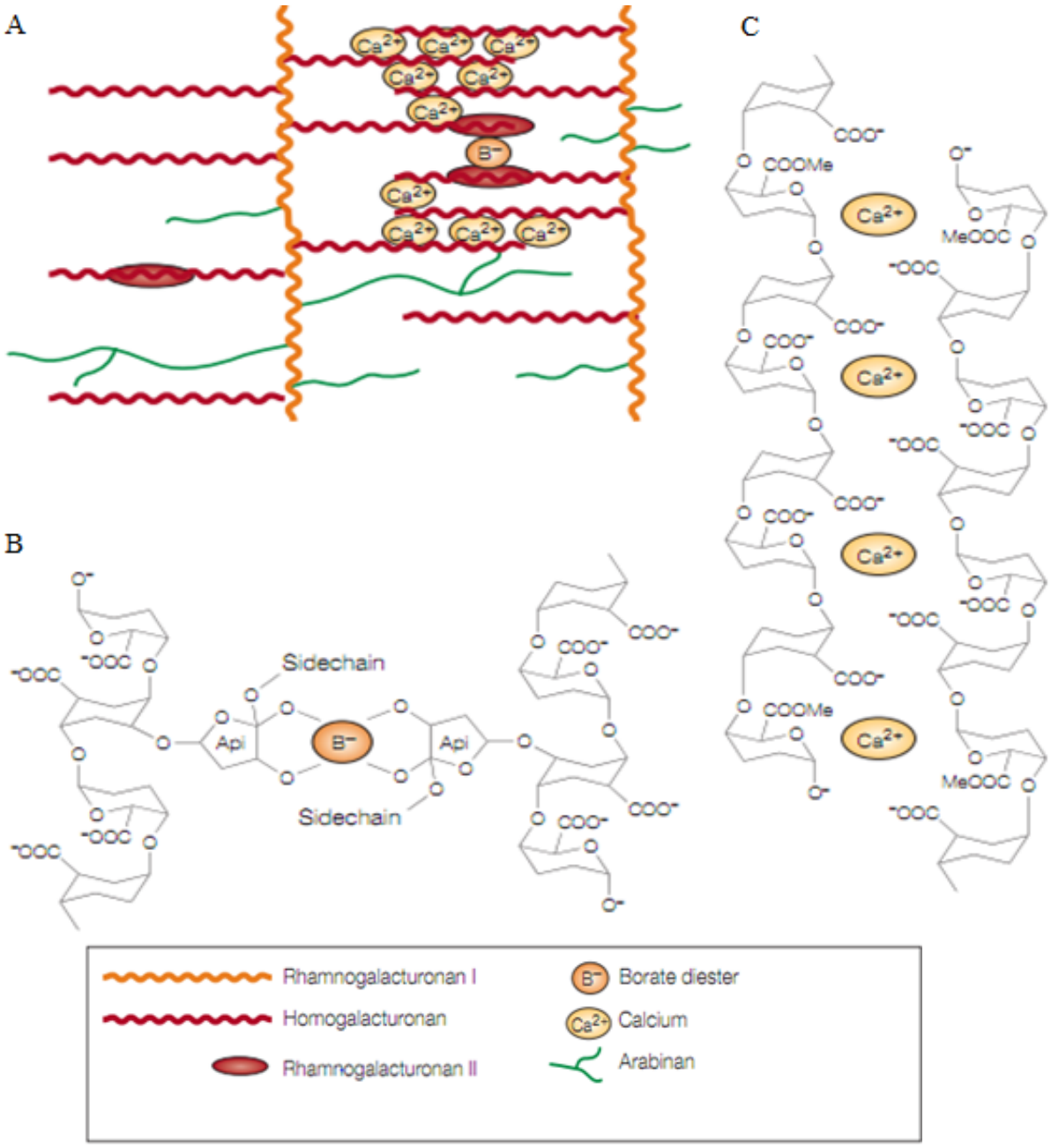


Figure 2.3 The formation of pectin networks by covalent and ionic bonds (Cosgrove, 2005). A shows how the pectin domains are covalently linked together to form a massively large macromolecular pectin network. This is a simplified version of a recent model by Vincken *et al*. (2003), in which rhamnogalacturonan I serves as the backbone and the other pectin domains are attached as branches. Homogalacturonans are ionically crosslinked by calcium (C) whereas boron crosslinked rhamnogalacturonan II through diester linkages (B).

rhamnogalacturonan I (RG-I) (O'Neill *et al*., 1990; Ridley *et al*., 2001; Caffall and Mohnen, 2009; Harholt *et al*., 2010). Homogalacturonan (HG) is an unbranched chain of α-1,4-linked-D-galacturonic acid (Gal*p*A) residues, which is the main type of pectins in plant cell walls and the middle lamella, occupying more than 60% (Ridley *et al*., 2001). Rhamnogalacturonan-I (RG-I) is composed of a backbone in which the disaccharide [4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p*] is continuously repeated (Ridley *et al*., 2001). The backbone of Gal*p*A residues can be substituted at various positions by β-D-xylosyl (Xylp) residues and β-D- apiofuranosyl (Apif), forming xylogalacturonan (XGA) and apiogalacturonan (AGA), respectively (Hart and Kindel, 1970; O'Neill *et al*., 1990; O'Neill *et al*., 2003; Zandleven *et al*., 2006; Caffall and Mohnen, 2009). Although rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I) have similar names, their structures are not related. The basic backbone of RG-II are 1,4-linked α-D-Gal*p*A residues and there are four structurally different oligosaccharide side chains linked to the RG-II backbone.

Matsuura *et al*. (2000) extracted the onion (*Allium cepa* L.) pectins and found pectins from onion contain 93.7% uronic acid, indicating the main composition of onion pectin is homogalacturonan (HG). There is also a small rhamnogalacturonan (RG) content, approximately 2.3%. Therefore, HG is very critical in onion cells. Expanding cells usually synthesize homogalacturonan which is highly methyl esterified. The degree of HG methyl-esterification plays an important role in the developmental processes of plant organs such as cell division, expansion, and adhesion (Shevell *et al*., 1994; Wolf *et al*., 2009). Since the un-methylated Gal*p*A residues are anions, the un-methylated galacturonic acid residues can ionically cross-link with Ca^{2+} to form a stable gel with other pectic molecules, forming an egg-box model structure (Figure 2.3 C), which greatly increases cell wall texture and mechanical properties (Grant *et al*., 1973; Liners *et al*., 1989; Caffall and Mohnen, 2009; Wolf *et al*., 2009). By contrast, although RG II molecules also can form cross-linkages, it forms covalent bonds via borate esters (Figure 2.3 B) and not ionically with calcium (Matoh and Kobayashi, 1998; Ridley *et al*., 2001). This cross-linkage is important in the formation of normal cell walls, controlling wall porosity and wall thickness (Cosgrove, 2005). The Ca^{2+} cross-bridges condense part of the HG to create pores and contribute to the reduction in cell wall porosity (Moore and Staehelin, 1988; Knox *et al*., 1990). The un-methylesterified HG results from the hydrolysis of the methyl ester bond of HG Gal*p*A residues under the catalysis of pectin methylesterase (PME) secreted into the

extracellular wall space, altering the degree while pattern of methyl esterification and releasing methanol and protons (Jolie *et al*., 2010).

2.2.2 Function of the Apoplast

In general, the cell wall is a physical barrier that has several specific functions:

(1) Maintain or determine cell shape and size to give cells a definite structure for formation of tissues and organs in plants (Geitmann, 2010). Control the rate and direction of cell growth and regulate cell volume, thereby achieving the purpose of regulating cell expansion (Bashline *et al*., 2014).

(2) Separate interior of the cell from the outer environment and help the cell-cell communication (Keegstra, 2010) through the plasmodesmata (specialized pores within the cell wall) (Raven, 1997) or signaling molecules located in the cell wall such as arabinogalactan proteins (Seifert and Roberts, 2007).

(3) Constrain the turgor pressure, preventing the cell from rupturing due to tugor pressure (Knox, 2008).

(4) Act as a physical barrier to biotic and abiotic stresses, provide protection from infection and mechanical stress, and prevent water loss (Albersheim and Anderson-Prouty, 1975; Bateman and Basham, 1976). The cell wall-degrading enzymes which are secreted by microbes can be inhibited by cell wall proteins; some enzymes within cell walls have the ability to degrade the microbes. As a result, the invasion of microorganism can be prevented by the first barrier—the cell wall (Albersheim and Anderson-Prouty, 1971; Cline and Albersheim, 1981; Laluk and Mengiste, 2010). Alterations of the structure and composition of the plant cell wall also play a crucial role in the defense mechanism against low temperature (Rajashekar and Lafta, 1996; Murai and Yoshida, 1998; Jones *et a*l., 2000; Solecka *et a*l., 2008), drought (Leucci *et al*., 2008; Jiang *et al*., 2012), salt stress (Uddin *et al*., 2013; de Lima *et al*., 2014), etc.

(5) Provide carbohydrate storage for other metabolic processes such as seed germination (Meier and Reid, 1981; Brett and Waldron, 1996; Buckeridge *et al*., 2000). The storage cell wall compounds in seeds include xyloglucans, pectic galactans, hemicellulose etc., which are mainly localized in the endosperm or cotyledons (Brett and Waldron, 1996). For example, under the low light conditions, the high xyloglucan content which exists in the cotyledons is used as the carbon source for the germination of *Hymenaea courbaril* (*Leguminosae*) seeds

(Santos and Buckeridge, 2004).

(6) As economic products – many industrial products depend on cell wall material, such as timber, pulp, paper, wood, fiber, cellulosic biofuel, shelter, and even food (McNeil *et al*., 1984; McDougall *et al*., 1996; Bashline *et a*l., 2014).

2.2.3 Responses to Cold Acclimation and Role in Freezing Resistance

As the primary physical barrier, numerous changes including the structures and compositions of cell wall are induced in response to freezing stress after a period of cold acclimation. During freezing, cell dehydration reduces cell volume but the cell wall is capable of resisting this reduction, preventing water loss and ice penetration to the intracellular spaces (Rajashekar and Burke, 1996). Dehydration resistance may lead to tightening and loosening of cell walls (Moore *et al*., 2008b) and extensive cell wall folding as seen in C. *plantagineum* (Hartung *et al*., 1998; Farrant, 2000) in order to avoid water loss inside cells. Water initially crystalizes in the extracellular space in plant tissues under subzero temperatures (Levitt, 1972). The plasma membrane may be damaged by the cell walls because of the mechanical distortion caused by the external ice crystals (Iljin, 1933). The ability of Jerusalem artichoke to resist freezing is enhanced through cold acclimation, although the influences are negative when the attachment between plasma membrane and cell wall is tight (Murai and Yoshida, 1998).

The apoplast can contribute to freezing resistance by physically preventing ice from nucleating the intracellular space. The quantity of cell wall increases in cold-acclimated pea seedlings (Weiser *et al*., 1990), bromegrass cell suspension cultures (Tanino *et al*., 1990) and leaves of winter rapeseed (Kubacka-Zebalska and Kacperska, 1999; Solecka *et al.*, 2008), contributing to cell wall thickening to a certain degree (Solecka *et al.*, 2008). Reports of cell wall thickening during cold acclimation are consistent with the idea that altered cell wall elasticity is considered an important component of the adaptive response (Hüner *et al*., 1981), which is thought to affect water movement and in terms of freezing resistance, related to the pattern of ice propagation in the cold-acclimated plant tissues (Griffith and Brown, 1982; Griffith *et al*., 1985). Cell wall thickening is also observed in the leaves of potato (Chen *et al*., 1977), cold-hardy rye cv. Puma (Huner *et al*., 1981; Griffith and Brown, 1982) and winter oilseed rape (Stefanowska *et al*., 1999) in response to cold acclimation. Cell wall strength increases and the pore-size of the cell wall decreases in grape and apple cells upon cold acclimation (Rajashekar and Lafta, 1996).

Increased cell wall rigidity has been proposed to protect cells against intracellular freezing (Rajashekar and Lafta, 1996).

Alterations in cell wall composition, the activities of cell wall-modifying enzymes, and the accumulation of particular proteins in the apoplast space in plant tissues are induced during cold acclimation (Weiser *et al*., 1990; Marentes *et al*. 1993, Hon *et al*. 1994, Antikainen *et al*. 1996). The water soluble carbohydrate compositions including fructan, sucrose and fructose of cereal crops also changes during exposure to freezing temperatures, in which fructan decreased while fructose and sucrose increased (Olien and Lester, 1985; Olien and Clark, 1993). As a result of fructan hydrolysis, the increase in sugars in the apoplast is thought to be one of the reasons for the improved hardiness during subzero acclimation (Olien and Lester, 1985; Livingston and Henson, 1998). The presence of cell wall polymers reduces the growing speed of ice crystals in the middle lamella (Olien and Smith, 1977). Studies have demonstrated that the epoxide monomer, lipid-derived polymers cutin and suberin are deposited and can be visualized in the mestome sheath cell walls (Kolattukudy, 1980). Moreover, cold acclimation increases hemicellulose content (Weiser, 1990; Kubacka-Zębalska and Kacperska, 1999) and reduces xylose and glucose contents in winter oilseed rape leaves (Kubacka-Zębalska and Kacperska, 1999). Cold acclimation also affects the accumulation of cell-wall proteins and the activities of cell-wall enzymes. Extensin, one of the structural cell wall glycoproteins which are thought to provide rigidity to the cell wall by forming an interpeptide-linked network (Lamport, 1967), increases in cold acclimated pea along with an increase in freezing resistance (Weiser *et al*., 1990). Two glycine-rich proteins encoded by *MsaCiA* and *MsaCiB* mRNAs accumulate in both leaf and crown cell walls of cold acclimated alfalfa (Ferullo *et al*., 1997). Numerous other studies have focused on antifreeze proteins (AFPs) which accumulate in the mesophyll cell walls, secondary cell walls of xylem vessels and epidermal cell walls of plants during cold acclimation (Griffith *et al*., 1997). Cold acclimation increases both transcriptional and translational level of AFP genes (Yeh *et al*., 2000; Huang and Duman, 2002). AFPs can adsorb onto ice crystals and prevent ice growth (Raymond and DeVries, 1977). AFP activity is present only in the apoplastic extracts from freezing tolerant monocotyledons after cold acclimation (Antikainen and Griffith, 1997), where they enhance freezing resisting (Griffith and Yaish, 2004; Griffith *et al*., 2005). Apoplastic AFPs modifies the normal growth pattern of ice crystals in winter rye after cold acclimation and endures extracellular ice during freezing stress (Griffith *et al*., 1992; Hon *et al*.,

1995). The study by Griffith *et al*. (1992) illustrates that proteins with antifreeze-like properties from the apoplast of acclimated rye leaves have antifreeze activity and these protein mixtures modify the growth pattern of ice crystals. Interestingly, AFPs do not exhibit antifreeze activity in non-acclimated plants even though they are present within those plants (Wang *et al*., 2006). The antifreeze activity of AFPs is activated after exposure to low temperature and is only present in plants which can tolerate the presence of ice in their tissues (Yaish *et al*. 2006). Some cell-wall degrading enzymes are also modified by cold acclimation. For instance, the activity of pectin methylesterases (PMEs) was enhanced during cold acclimation in winter oilseed rape (Solecka *et al*., 2008) and in cold-treated Arabidopsis (Lee, 2003). This enzyme is capable of regulating the degree of pectin methyl esterification through catalysing the demethylation reaction of methylesterfied pectin to form the un-methylesterified pectin, affecting the cell wall rigidity.

These observed changes in cell wall compositions are associated with the regulation of genes encoding proteins involved in cell wall modification (Zabotin *et al*., 1998). The same genes may be regulated in various ways at low temperature. For example, the expression of the *EXP* genes (expansin genes which modulate cell-wall loosening and their activities can be affected by drought (Jones and McQueen-Mason, 2004) and salt stress (Buchanan *et al*., 2005)). During cold acclimation, *EXP* genes are down-regulated in *Arabidopsis* roots (Swindell, 2006) and sweetpotato (Noh *et al*., 2009), but up-regulated in *Arabidopsis* seeds and rice anthers (Imin *et al*., 2004; Yamauchi *et al.*, 2004). Both *EXP* and *XTH* (xyloglucan endotransglucosylase / hydrolase genes), which regulate cell wall xyloglucan content and plant growth (Miedes *et al*., 2013), are known to improve resistance to drought, salt and aluminum stresses (Han *et al*., 2013). *EXP* and *XTH* genes in sweetpotato, Extensin and *XTH* encoding genes in *Arabidopsis* are down-regulated under cold response, consistent with the improvement in freezing tolerance (Seki *et al*., 2002). Pectin methylesterases (PMEs) located in the cell walls are encoded by a multigene family (Richard *et al*., 1996; Micheli *et al*., 1998), and 67 *PME*-related genes have been identified from the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). *PME* gene expression or its activity is generally thought to be strongly associated with dormancy breakage (Ren and Kermode, 2000), seed germination (Müller *et al*., 2013), tube pollen growth (Wakeley *et al*., 1998; Futamura *et al*., 2000), and fruit maturation (Kagan-Zur *et al*., 1995). In addition, Zhu *et al*. (2013) demonstrated a PME3 in cold acclimated cotton is up-regulated. Arabinogalactan proteins (AGPs) located in plant cell walls, which are involved in plant growth

and development, are also up-regulated in response to cold stress during early root development of cotton (Gong *et al*., 2011). Although numerous researchers support cell walls functions as a barrier and provides freezing resistance, relatively little work has been done to identify those specific components within the cell wall and the apoplastic space that are critical to this resistance.

Pectic polysaccharides are connected to the integrity and rigidity of plant tissue, cell wall porosity (Baron-Epel *et al*., 1988) and abiotic and biotic stress resistance (Hahn *et al*., 1989; Vorwerk *et al*., 2004), affecting the tissue ability to supercool (Ashworth and Abeles, 1984). In the study of Solecka *et al*. (2008), the tensile strength of a leaf tissue was positively associated with pectin content and its degree of methyl esterification after cold acclimation. Cell wall porosity and gelation is generated via the formation of the pectic gel resulting from ionically cross-linking un-methylesterified HG and Ca^{2+} and the degree of pectin methyl-esterification (Grant *et al*., 1973; Yoo *et al*., 2003). Cell wall porosity is related to the resistance of plant cells to low temperature damage. For example, the pore size of the cell wall in both grape and apple cell cultures decreased and the cell wall strength increased after cold acclimation (Rajashekar and Lafta, 1996). The ultrastructural observation of peach and dogwood cells demonstrated that both pit membrane (black cap and primary cell-wall) and xylem parenchyma cells were almost completely digested by the treatment of macerase (an enzyme mixture rich in pectinase), resulting in a complete shift of the low-temperature exotherm (LTE) to warmer temperature as determined by differential thermal analysis (DTA) (Wisniewski *et al*., 1991a). Thus, pectins appear to regulate water and ice movement through vascular pits thereby enhancing deep supercooling (Wisniewski *et al*., 1991a). This result was further confirmed by the study from Jones *et al*. (2000) in which a 3000 MW dextran-conjugated dye cannot penetrate the bud axes after cold-acclimation, indicating that this lower porosity probably restricts the penetration of ice into buds from the grape cane. In addition, Jones *et al.* (2000) found apoplastic permeability increased because of the treatment of pectinase which removes pectin, and increased the ice nucleation temperature, preventing supercooling according to the results from DTA. Using FTIR, Wilson and his co-workers (2000) indicated macromolecules in onion cell walls such as cellulose and pectin are reoriented along the direction of cell elongation (parallel) from a small directional alignment with parallel and perpendicular polarizations after going through a mechanical stress.

The pectin content increases in pea epicotyls (Weiser, 1990), winter oilseed rape leaves (Kubacka-Zębalska and Kacperska, 1999; Solecka *et al*., 2008) and bromeliad (*Nidularium minutum*) (Carvalho *et al*., 2013) during cold acclimation. In the frost-tolerant pea genotype, a period of cold acclimation induced an increase in HG (Baldwin *et al*., 2014). The degree of pectin methylesterification is regulated by the pectin methylesterases (PMEs). PME activity increases in cold-acclimated oilseed rape leaves, which was associated with the improved cell wall rigidity caused by de-methylesterification of pectin. More un-methylesterified pectin was formed as a result of higher PME activity, creating a semi-rigid pectate gel through the Ca^{2+} cross-linking with free carboxyl groups (Jarvis, 1984; Carpita and Gibeaut, 1993; Solecka *et al*., 2008). Therefore, the enhanced PME activity could improve cell wall stiffness and increase freezing tolerance of plants. In addition, as 'pectic plasticizers', the arabinose polymers can hold the flexibility of cell walls during dehydration and rehydration of guard cells (Jones *et al*., 2003) and aquatic plants (Moore *et al*., 2008a) and thus may protect cells from damage in the process of freezing-thawing.

2.3 Calcium

2.3.1 Calcium is an Essential Element in Plants

Calcium is one of 14 essential nutrients in plants and plays important roles in structural support of the cell wall and plasma membrane, growth and signal transduction in plant tissues (Maathuis, 2009). As the cellular structural supporter, the cation Ca^{2+} can form the complexes with anion groups of organic compounds including phosphates and carboxyl of phospholipids, proteins and sugars. In the process of cell wall formation, Ca^{2+} is a crucial element to determine the structural rigidity of the cell wall since Ca^{2+} can electrostatically form cross linkages to the free carboxyl groups of acidic pectin residues which are released by hydrolysis of the methyl-esterified pectin under the catalysis of PME (Wyn Jones and Lunt, 1967; Burström, 1968; Hepler, 2005). Calcium in plant cells mainly localizes in the endoplasmic reticulum (about 1mM) and the apoplast (around 1-10mM) (Bush, 1995; Trewavas and Malho, 1998). The high apoplastic Ca^{2+} concentration, up to 60-75% of the total tissue Ca^{2+} content (Demarty *et al*., 1984), makes the cell wall more rigid and less plastic, helping to increase biotic and abiotic stress resistance (Hepler, 2005). In the plant plasma membrane, Ca^{2+} complexes with phosphate groups from

phospholipids in order to maintain the integrity and permeability of the plasma membrane (Hepler, 2005; Maathuis, 2009).

Calcium is necessary at all stages of plant growth and development including the regulation of growth and differentiation in cells and tissues (Hepler and Wayne, 1985; Trewavas and Malho, 1998; Sanders *et al*., 1999), embryogenesis (Medvedev, 1997) etc. Ca^{2+} also acts as a secondary messenger. Changes to the cytosolic free Ca^{2+} in plants are induced by the biotic and abiotic stress, stomatal regulation and physical damage (Mahouachi *et al*., 2006; McAinsh and Pittman, 2009). Research has demonstrated that Ca^{2+} influx is temporally moved into the cell cytoplasm under the stimuli of the stress signal, inducing a transient increase in cytosolic Ca^{2+} which then activates the cascades of phosphoproteins like calmodulin and finally induces the expression of stress-responsive genes such as antifreeze proteins (AFPs) during freezing stress (Sanders *et al*., 1999; Knight, 2000, Xiong *et al*., 2002).

2.3.2 Role of Calcium in the Apoplast and Freezing Stress Resistance

Today, no one questions that Ca^{2+} is a crucial factor in regulating the growth and development of plants. Through coupling of extracellular signals and intracellular second messengers, Ca^{2+} participates in various physiological and biochemical reactions such as controlling plant growth and differentiation (Hepler and Wayne, 1985), regulating embryogenesis (Medvedev, 1997), assembling and disassembling of cytoskeleton elements (Medvedev and Markova, 1998), and modulating movement of stomatal cells (Ng *et al*., 2001). Thus, Ca^{2+} plays vital roles in the whole life cycle of plant (Medvedev, 2005). Intracellular levels of calcium ions are significantly modulated following a plant's exposure to either abiotic or biotic stresses (Ludwig *et al*., 2004). For example, cold stimuli can induce a rapid increase in plant cellular Ca^{2+} level (Knight *et al.*, 1996). The cold resistance in grafting eggplant seedlings after the application of exogenous Ca^{2+} is higher than those with calcium deficiency (Gao *et al*., 2004).

Since the 19th century, Ca^{2+} has been known to play a crucial role in determining the structural rigidity and stabilization of the cell wall (Wyn Jones and Lunt, 1967; Burström, 1968) through forming calcium bridges with pectin molecules which is regulated by the activity of PME (Chang *et al*., 1995). Suutarinen *et al*. (2000) used instrumental textural measurements, as well as microscopic studies to show that in most cases, $CaCl_2$ pre-freezing treatments stabilized the structures including cell walls (in particular pectin) of strawberry tissues. Two areas within

the cell have been recognized as being important targets for Ca^{2+}: the cell wall and endoplasmic reticulum. In terms of the cell wall, calcium is a major pectin cross-linking agent which results in increased gel density, reduced cell wall porosity and increased cell wall rigidity (Peaucelle *et al*., 2012). Therefore, we propose that the dynamic increase in pectin gelation which is driven by PME activity and calcium binding during cold acclimation provides cells ability to avoid freezing damage through a physical blockage of ice crystals from nucleating the intracellular space.

2.4 *Allium fistulosum* L.

Allium fistulosum L. (2n =2x = 16) (Inden and Asahira, 1990), known as Japanese bunching onion or Welsh onion, is a perennial plant and belongs to the genus *Allium* L. in the section *Cepa* (Mill.) Prokh (Hanelt, 1990) and is one of the most important cultivated edible *Allium* crops (Others are onion, leek, garlic.). Other names that may be applied to this plant include green onion, spring onion, scallion, and salad onion. It is thought to have originated in northwestern China and is a leaf vegetable mainly cultivated in East Asian countries, such as China, Japan and Korea, as well as in European countries (Grevsen, 1989; Ford-Lloyd and Armstrong, 1993). The Japanese onion contains abundant vitamin C (31-36%) (Kołota *et al*., 2013), but also flavonoids (283 mg/kg Fresh Weight) (Aoyama and Yamamoto, 2007), macro- and micro-nutrients, especially Ca (1%) and K (3%) (Kołota *et al*., 2013). Most onions can multiply by forming perennial evergreen clumps (Thompson, 1995).

Japanese bunching onion is vigorous with a rapid growth rate (Lazić *et al*., 2002; Štajner *et al*., 2006) and resists summer drought, winter cold and poor soil quality (Yamasaki *et al*., 2003). Some Japanese bunching onions can survive when the air temperature drops below -40℃. The species also has the resistance ability against onion blight, pink root (Netzer *et al*., 1985), and other plant diseases and insect pests. Onion has a unicellular epidermal layer (Wilson *et al*., 2000) located at the base of the leaf and is easy to peel. In addition, the individual cell size of Japanese bunching onion is quite large, approximately 250 μm in length x 50 μm in width x 90 μm in thickness. The epidermal cell layer has no chlorophyll, avoiding issues of auto-fluorescence during cell studies based on fluorescent probes. Therefore, *Allium fistulosum* (Japanese onion bunching) is an ideal system to observe freezing processes.

In 1956, Asahina (1956) conducted freezing tests on the epidermis of unhardy and hardy Welsh onion leaves. He found that the unhardy cells became immediately opaque and dark and then lighter once the temperature decreased to below the freezing temperature, and the ice would rapidly propagated the whole protoplast. In contrast, the freezing rate in epidermal cells was reduced and the darkening phenomenon in frozen cells was lowered if the epidermal cells are hardy or have a certain degree of desiccation. In those studies, intracellular freezing (at -13.5^{o}C) in hardy epidermis of Welsh onion was markedly later than extracellular freezing (at -7^{o}C). These interesting observations led us to study the mechanism of intracellular freezing stress avoidance on *Allium fistulosum*.

3.0 MATERIALS AND METHODS

3.1 Plant Materials and Growth

Japanese bunching onion (*Allium fistulosum L.*) seeds produced from plants growing outdoors in Saskatoon, SK (52°7'N) were collected and germinated in the College of Agriculture and Bioresouces greenhouses (45 Innovation Blvd., University of Saskatchewan, Saskatoon, SK Canada). Plants were grown for 3 months in 6" pots of soilless mix (Sunshine No. 4, Sungro Hort Inc, Bellvue, WA) at (20 ± 5°C) under natural light supplemented with 400 W high-pressure sodium lights (18 h photoperiod, average of 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (see Appendix A). Plants were irrigated once every second day and fertilized weekly with 20-20-20 (NPK, 2 $g \cdot L^{-1}$) + micronutrients including boron, copper, iron, manganese, molybdenum, zinc (Plant Products Co. Ltd., 314 Orinda Road, Brampton, Ontario, LGT-1J1). For calcium treatment, each plant was watered with 100 ml 0.05 M $CaCl_2$ solution every second day over a four-week periods in the greenhouse. Subsequently, half of the treatments plus and minus calcium were transferred into a Conviron growth chamber with 12°C/4°C (day/night) under an 8 hour photoperiod with light intensity of 370 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for two weeks for cold acclimation treatment. In all, there were four different treatments: Ca (-) non-acclimated (NA), Ca (+) non-acclimated (NA+Ca), Ca (-) acclimated (ACC), Ca (+) acclimated (ACC+Ca).

3.2 Freezing Stress Treatments

The leaves of onion were numbered from 0 to 4 in order of age in which 0 is the youngest leaf and 4 is the oldest leaf (Figure 3.1, also see Appendix I). In grass, the collar region is located between the leaf blade and leaf sheath, containing structures called ligules which is the membranous tissue located at the junction of the leaf blade and leaf sheath (Figure 3.2) (Harper and Freeling, 1996). The ligule is also called epidermal layer which is easily peeled off from onion and kept as an intact single cell layer, simplifying sample preparation. Bin Li previously performed the freezing test among different ages of leaves and different regions of the same ligule (Figure 3.1). More specifically, the freezing tolerance in the leaves was found to be leaf0 > leaf1 > leaf2 > leaf3 > leaf4. The region C-b located between 2 and 3 cm from the ligule in the center of the sheath epidermal cell layer (Figure 3.1 B) is more freezing stress resistant than other regions of the same epidermal layer (Tanino *et al*., 2013). However, the region B-b (located

between 1 and 2 cm from the ligule in the center of the sheath epidermal cell layer) in leaf2 located in the center of the various regions expressed greater consistency of freezing stress resistance and was selected in this study. Leaf 2 of each sample (NA, NA+Ca, ACC and ACC+Ca) was placed into a 50 ml glass tube with 2 ml distilled water, and cooled to -2.5°C for 2 hours using an Endocal RTE-Series Refrigerated Bath. Thereafter, ice was added to the tube to nucleate the sample. After one hour, the temperature was decreased at a rate of -5°C·hour^{-1}, held for one hour and the first sample was removed. Samples were removed at -10°C, -15°C, -20°C, and -25°C, -30°C. Upon removal from the refrigerated bath, the samples were placed into a 4°C refrigerator overnight to thaw. The next morning, samples were warmed to room temperature (24°C) for at least one hour before observation. The epidermal layer of each sample was peeled and fixed onto the slide with a coverslip. Presence or absence of protoplasmic streaming was used as a measurement of the number of live and dead cells (10 cells per sample, 3 samples per replication, and 5 replications per temperature).

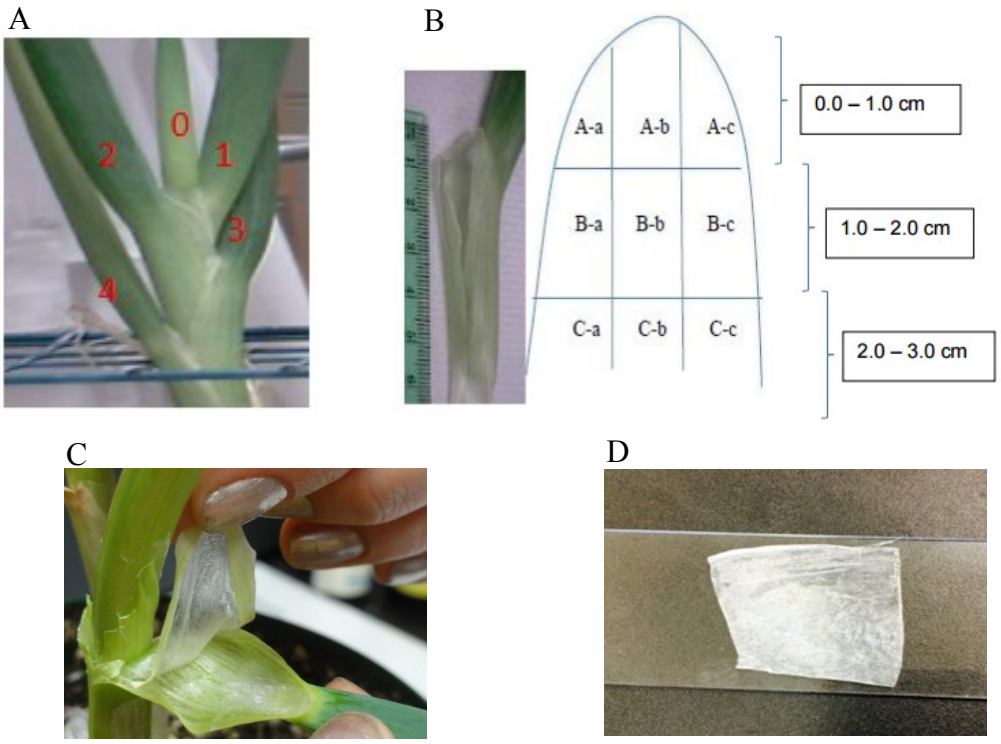


Figure 3.1 The experimental area of Japanese bunching onion. A: numbering system of the whole plant according to leaf age; B: numbering system in one ligule; C: the ligule (or epidermal layer) separated from the sheath; D: the ligule epidermal layer placed on a glass slide.

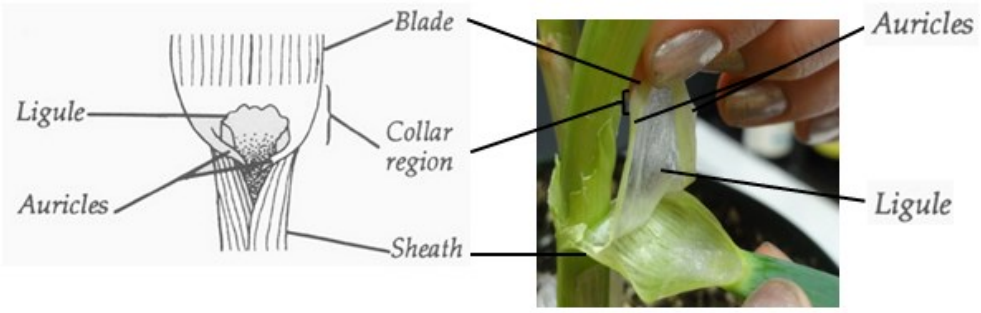


Figure 3.2 The ligule and collar region of the plant. Right: the classical structures of ligule in Poaceae (http://weeds.cropsci.illinois.edu/weedid.htm). Left: the ligule in *A. fistulosum*.

3.3 Analysis of Freezing Process

The freezing process including ice nucleation in extra- and intra-cellular space was recorded with a Nikon E400 microscope in Department of Food and Bioproduct Sciences, University of Saskatchewan. The cryostage apparatus (Linkam freezing stage, LTS 120, UK) was connected to a Nikon E400 light microscope with a camera attached (Nikon Digital Sight). The epidermal layers of leaf 2 from control and treated onions were peeled and the abaxial surface placed down onto the glass slides. The slide was fixed on the cryostage. Before lowering the temperature, protoplasmic streaming of live cells for viability analysis was confirmed and video recorded at 24°C. The cryostage was water-cooled using a heat sink pump and the cooling rate was adjusted by a controller (Linkam PE94, UK). The temperature was decreased to 0°C and finally to -23°C (the lowest temperature achieved by the heat sink) at a rate of $5°C \cdot hour^{-1}$. The process of ice crystal formation in the cells was video-recorded in real time with Cam Studio using a Nikon 40X lens (see Appendix B). When ice formation occurred, the refraction of light changed and the temperature at which extra- and intra-cellular ice nucleation occurred was recorded. Samples were then thawed at a rate of $1°C \cdot min^{-1}$ and again examined for the presence of protoplasmic streaming (see Appendix C) when temperature reached 24°C. Treatments also included ice nucleation (+) induction by adding a few grains of silver iodide powder (AgI) resuspended in water to the glass slide. In these experiments, the onion epidermal layer was placed on top of the silver iodide. The temperature was then decreased to -2.5°C and held for two hours to further initiate ice nucleation. Each treatment was replicated five times.

3.4 Study of Apoplast Permeability

The epidermal layers of non-acclimated (NA) and acclimated (ACC) onions were

submerged in the mixture of fluorescein conjugated to a 3,000 MW and 70,000 MW dextran solution (5 $mg \cdot ml^{-1}$ in 0.1 M phosphate buffer, Life Technologies) for 16 h and observed by a Laser Scanning Confocal Microscope (ZEISS LSM 510 Confor2, Department of Biology, University of Saskatchewan, see Appendix E).

The diffusion rate of fluorescein (Sigma-Aldrich Chemical Co. St. Louis, MO) from the extracellular to the intracellular space was measured using a Laser Scanning Confocal microscope (LSM, Nikon Eclipse 80i Microscope, see Appendix E). The epidermal layers of non-acclimated and cold acclimated onions were peeled and flattened onto the cover well with glass wool (the adaxial side was toward the glass wool), and then covered with a glass slide. After sample preparation, the slide was placed into the LSM. Next, the 100 µl 1 $mg \cdot ml^{-1}$ fluorescein solution was slowly injected through a hole in the cover well to soak. The focus was kept approximately 40 µm which roughly corresponds to the midpoint of the intracellular space (total onion cell thickness is 90 µm). The diffusion process of fluorescein in individual samples were recorded every 10 seconds by the NIS-Elements imaging software. In order to calculate the diffusion rate of the samples, the NIS-Elements software was used to select the region of interest (ROI) with an intact cell including the apoplastic space, plasma membrane and cytoplasmic region. The ROI in the samples was scanned and the time at which the pixel brightness increased to 2000 pixels (mean intensity) at that location was recorded. The diffusion rate was calculated based on the slope with pixels as the Y axis and time as the X axis.

3.5 Analysis of Pectin Methyl Esterification

3.5.1 Sample Preparation and Fixation

The sample preparation was based on Lee *et al*. (2008). The onion whole sheath was cut into 2-3 mm pieces and fixed in 0.01 M sodium phosphate buffer (including 1% formaldehyde and 0.025% glutaraldehyde, pH 7.2). The samples were infiltrated with vacuum for a minimum of 1 h in order to thoroughly fix the samples and then incubated at 4^{o}C overnight. The fixed samples were then successively washed with 0.01 M sodium phosphate buffer twice for a total of 4 hours and dehydrated in a graded ethanol series (30, 50, 70, 95, and 100%) at one hour for every concentration of ethanol at 4^{o}C. The samples were then infiltrated with London Resin (LR) white (Electron Microscopy Sciences, https://www.emsdiasum.com):ethanol (1:1) for 5 hours at 4^{o}C, and this step was repeated once overnight. The samples were subsequently transferred into a

2:1 concentration of LR white resin: ethanol for 7 hours and then overnight. The last infiltration step with pure LR white resin was added to the samples at least 6 times and changed every 12 hours. Finally, the samples were transferred into an embedding mold and polymerized at 60^{o}C for 24 to 48 hours. The samples were sectioned after embedding using the Reichert-Jung Ultracut Microtome equipped with a diamond knife (Department of Biology, University of Saskatchewan). Sections (1 μm thick) were placed on poly-L-lysine coated slides (Electron Microscopy Sciences, https://www.emsdiasum.com) and heated on a 55^{o}C warm plate to adhere the sections to the slide.

3.5.2 Analysis of Pectin Methyl Esterification by Immunohistochemistry

The immunofluorescence labelling analysis to evaluate the degree of pectin methyl esterification was conducted with the antibodies JIM5 recognizing de-methylesterified epitopes of HG and JIM7 recognising methylesterified epitopes of HG (CarboSource Services, http://www.ccrc.uga.edu/~carbosource/CSS_home.html). These experiments were conducted in the Norwegian University of Life Sciences Imaging Lab, Ås, Norway and subsequently repeated three times in the University of Saskatchewan. The experimental procedure followed Lee *et al*. (2008). The 1 μm thick sections obtained as described above were processed and were first incubated in 1 ml 3% (w/v) milk protein in phosphate-buffered saline (PBS), pH 7.2, for at least 30 min to block non-specific binding sites. Thereafter, sections were incubated with primary rat monoclonal antibodies JIM5 and JIM7 diluted 1:10 in PBS, respectively, for at least 1 h at room temperature and then washed three times with PBS at least 5 min for each time. Next, the secondary anti-rat-IgG+IgM+IgA antibodies linked to fluorescein isothiocyanate (FITC; Abcam) were diluted 100-fold PBS and 200 μl was added to each slide. After 1 h incubation at room temperature, the sections were washed in PBS buffer three times at least 5 min for each time. Finally, the sections were mounted with prolonged gold antifade reagent (Life Technologies) and examined with a fluorescent microscope (Evos digital inverted microscope, see Appendix E). All incubation steps were operated in the dark. The blank control was only added to the secondary antibody in order to show that the label is specific to the primary antibody and the other steps followed above.

3.5.3 Determination of PME Activity

PME activity under four different treatments was performed according to Richard *et al*.

(1994). Fresh epidermal layers (0.2 g) of leaf 2, the non-epidermal layer (2 g) of leaf 2 and the whole sheath (including both epidermal layer and non-epidermal layer (2.2 g) of leaf 2 (see Appendix A) were homogenized in 0.05 M HEPES buffer, pH 6.5, containing 1X protease inhibitor cocktail (P9599, Sigma-Aldrich Chemical Co. St. Louis, MO), and washed several times with cold water. After air drying, crude cell wall preparation was used to extract cell wall proteins with 0.05 M HEPES buffer containing 1 M NaCl and 1X protease inhibitor cocktail, under gentle agitation at 4^{o}C for 2 h. Protein concentration in the extracts was determined by the method of Bradford (1976) using a spectrophotometer (GENESYS 10 Bio, Thermo Fisher Scientific, USA) and using different concentrations of bovine serum albumin (Sigma-Aldrich Chemical Co. St. Louis, MO) as standards. The prepared protein extracts from cell walls were used to determine PME activity.

In order to measure the PME activity, the reaction mixtures contained 0.5% (w/w) highly methylated citrus pectins (Sigma Chemical Co. St. Louis, MO), 0.2 M NaCl and 0.015 (w/v) methyl red as a pH indicator. The extract above (5 µl) was added to 1 ml of reaction mixture. Changes in colour from yellow to red (due to reduced pH during pectin de-esterification) were measured spectrophotometrically at 525 nm for 2 min at 25^{o}C. A calibration curve was generated by adding 1-200 nEq H^{+} from HCl to 1 ml of reaction mixture and the enzyme activity was calculated on the basis of cell wall protein. PME activity was expressed in PME units where one unit was defined as one nanoequivalent of protons (nEq H^{+}) released by milligrams of cell wall proteins during 1 min and recorded as a function of the original FW of that tissue.

The PME activity in the cooking onion (*A. cepa*) and green onion (*A. chinense*) were compared with Japanese bunching onion (*A. fistulosium*) and determined as described above.

3.6 Cell Wall Compositional Analysis

The cell wall compositional analysis followed the methods of Foster *et al*. (2010). These measurements were conducted in conjunction with RWTH Aachen University, Germany.

3.6.1 Cell wall isolation

The epidermal layers of non-acclimated and acclimated onions were peeled and freeze dried. Roughly 15 mg of sample was ground with 5.5 mm stainless steel balls in a 2 ml sarstedt screw cap tube for 1 min using a Retsch mill (Retsch Gmbh, Haan, Germany) operating at 25 Hz. After removing the steel balls, the ground samples were vortexed thoroughly with 1.5 ml 70%

aqueous ethanol and centrifuged at 10,000 rpm for 10 min to pellet the alcohol insoluble residue. The supernatant was decanted and 1.5 ml chloroform/methanol (1:1 v/v) solution was added to re-suspend the pellet by shaking the tube thoroughly. The mixture was then centrifuged at 10,000 rpm for 10 min and the pellet was re-suspended in 500 µl acetone. The solvent was evaporated to dryness at 35°C. In order to remove the starch from the cell wall, the pellet was re-suspended in 1.5 ml 0.1 M sodium acetate buffer (pH 5.0) and heated for 20 min at 80°C and then cooled on the ice. Next, the pellet was added with agents including 35 µl 0.01% sodium azide (NaN_3), 35 µl α-amylase (50 µg/1ml H_2O; from *Bacillus licheniformis*, Sigma-Aldrich Chemical Co. St. Louis, MO) and 17 µl pullulanase (1.25 g/ml, 18.7 units from *Bacillus acidopullulyticus*, Sigma-Aldrich Chemical Co. Inc. St. Louis, MO), and incubated overnight at 37°C in the shaker. The suspension was heated to 100°C for 10 min to terminate digestion before centrifuging at 10,000 rpm for 10 min. The supernatant containing solubilized starch was discarded. Meanwhile, the pellet was washed with 1.5 ml water three times and the pellet was re-suspended in 500 µl acetone. The prepared cell wall material was pelleted after air drying.

3.6.2 Matrix Polysaccharide composition

To determine the cell wall monosaccharide composition, 2 mg cell wall material (obtained as described above) was placed into 2 ml starstedt tubes and 20 µl inositol solution (5 mg/ml) was added as an internal standard. The cell walls were rinsed with 250 µl acetone to collect the cell wall material at the bottom of the tube and the acetone was evaporated very gently under airflow.

For the weak acid hydrolysis, each sample was mixed with 250 µl 2 M trifluoroacetic acid (TFA) and incubated for 90 min at 121°C. The sample was then cooled on ice and centrifuged at 10,000 rpm for 10 min. About 100 µl acidic supernatant, which contained the matrix polysaccharide derived monosaccharides, was transferred to 2 ml glass screw cap vials and the remaining pellet was used for the crystalline cellulose assay. The TFA was evaporated from the glass tube under gentle air. Then 300 µl 2-propanol was added, vortexed and evaporated at 25 °C. This step was repeated three times. Each dried sample had 200 µl of a sodium borohydride solution added since the reduction of the monosaccharides is the first step of the alditol acetate derivatization process. The fresh solution with 10 mg sodium borohydride per 1 ml of 1 M ammonium hydroxide was prepared every time and the glass vial was placed at room temperature for 1.5 hours. The solution was neutralized with 150 µl of glacial acetic acid before

vortexing and evaporating at 25°C. Then, 250 µl acetic acid/methanol (1:9 v/v) was added. After vortexing and evaporating at 25°C, 250 µl methanol was added and evaporated, and this process was repeated three times. Two chemicals including 50 µl acetic anhydride and 50 µl pyridine were added to the glass vial and incubated at 121°C for 20 min in order to make the acetylation of the alditols. The samples were cooled down to room temperature and the reagents were then evaporated from the samples by air flow. The next step of adding 200 µl toluene to evaporate, was repeated three times. The final steps extracted the alditol acetates. First, 500 µl ethyl acetate was added and slightly swirled. Second, 2 ml water was added and vortex very well. To obtain the clear separate layers, the mixture was centrifuged at 2,000 rpm for 5 min. Third, 50 µl of the ethyl acetate layer was diluted with 100 µl acetone and pipetted into GC/MS vials with inserts. Finally, the samples were injected into a GC equipped with a quadrupole MS (Agilent Technologies, Santa Clara, United States). A Supelco SP-2380 (30 x 0.25 mm x 0.25 µm film thickness) column was used with a 4 min solvent delay and a flow rate of 1.5 ml/min. Injected samples were subjected to the following temperature program: initial hold at 160°C for 2 min; a 20°C/min ramp to 200°C and hold for 5 min; a 20°C/min ramp to 245°C and held for 12 min; spike to 270°C and hold for 5 min before cooling to the initial temperature of 160°C. Peaks were identified by mass profiles and/or retention times of standards including fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid. Monosaccharides were quantified based on standard curves.

4.0 RESULTS AND DISCUSSION

4.1 Freezing Stress Resistance

Japanese bunching onions were acclimated in the growth chamber with 12/4 ^{o}C (day/night) for one- and two-weeks. The acclimation temperatures used in this project simulated the natural temperature changes in autumn in Saskatoon, SK Canada. In addition, the pre-test showed the leaves of Japanese bunching onion turned yellow under 8/4 ^{o}C (day/night) after four weeks acclimation, because onions in nature survive in winter through leaf dry down to re-mobilize carbohydrates to store in the bulb. Freezing resistance in *Allium fistulosum* epidermal cell layer increased under cold acclimation (ACC) (Figure 4.1). Presence of protoplasmic streaming was used as a viability test for live cells, whereas absence of protoplasmic streaming was interpreted to indicate cell death. After two weeks of cold acclimation under 12/4 ^{o}C (day/night), the LT_{50} (lethal temperature at which 50% of the cells were killed) of the epidermal cell layer of Japanese bunching onions decreased to -27^{o}C, compared to non-acclimated (NA) onions which had an LT_{50} value of -12^{o}C (Figure 4.1). This cold acclimation response was confirmed independently in Japan using the same source of *Allium fistulosum* but acclimated at a constant 2^{o}C for two weeks (Kobayashi *et al*., 2014). In the Japanese study, the vital stain, BCECF (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) was used to determine viability. BCECF is a membrane-permeable fluorescent indicator which can be converted into fluorescent products (Kobayashi *et al*., 2014). These products can either permeate into the cell freely by intracellular esterases or be retained by cells due to their negative charges (https://www.dojindo.com/Protocol/CellStaining_Protocol.pdf). Freezing stress resistance in this Japanese study also corresponds to our earlier work in which the *Allium fistulosum* epidermal cell layer was determined to be alive at -25^{o}C after two weeks of cold acclimation (Tanino *et al*., 2013). Therefore, using *Allium fistulosum* epidermal cell layers as a model for cold acclimation and freezing stress resistance appears to be reliable and consistent.

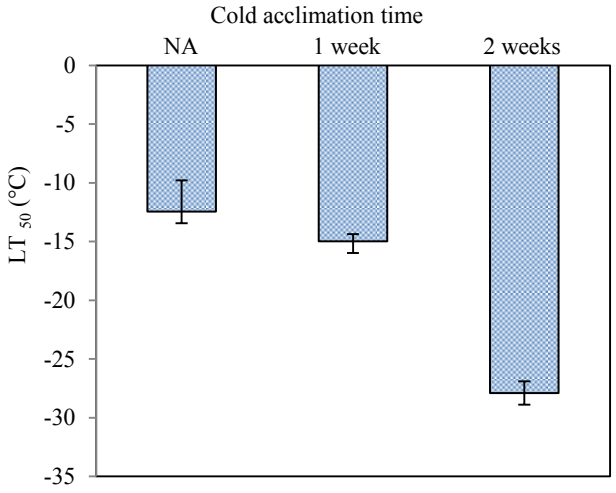


Figure 4.1 Effect of cold acclimation time on freezing resistance in non-acclimated (NA), 1-week and 2-week acclimated epidermal layer of *Allium fistulosum* L. (ACC). Cold acclimation was done using 12/4^{o}C, 8 hr photoperiod. Error bars are means ±SD based on the five separate experiments.

After the application of 0.05 M $CaCl_2$ solution as a soil drench for two weeks, the LT_{50} in non-acclimated onion cells (NA+Ca) was about -14^{o}C. As compared to no (minus) calcium treatment (NA) (-12^{o}C), and there was no significant difference (Figure 4.2). However, some NA+Ca cells were still alive at -20^{o}C in non-acclimated onions. In the absence of cold, a few reports have shown that the application of calcium alone can increase the freezing resistance in plants such as evergreen oak and apple (Percival and Barnes, 2008) and pear (Raese, 1997). All the cold acclimated samples in our study (including plus- and minus-calcium) had higher freezing stress resistance than non-acclimated onions (Figure 4.2). There was no significant difference in freezing stress resistance between ACC and ACC+Ca with LT_{50} values around -25^{o}C, -26^{o}C, respectively. However at lower temperature (-30^{o}C), the freezing stress survival of ACC+Ca was 44% higher than ACC alone (Figure 4.2). The positive role of calcium during cold acclimation are also shown by studies where the applications of EGTA (Ca^{2+} chelator), and La^{3+} and verapamil (Ca^{2+} channel blockers) inhibit protein phosphorylation, development of freezing tolerance, and reducing the accumulation of specific mRNAs in alfalfa during cold acclimation (Monroy *et al*., 1993). In red onions (*Allium cepa* L. cv Big Red), calcium is known to moderate the injury to the plasma membrane from freezing stress, and the application of exogenous calcium can also prevent cell plasmolysis and protoplasmic swelling resulting from the high concentration of extracellular K^{+} in freezing stress (Palta and Li, 1980; Arora and Palta,

1988; Palta, 1996). Mechanical stress, including mechanical deformation of the plasma membrane and the phase transition of the lipid compositions from the liquid to the solid state in plasma membrane (Yamazaki *et al*., 2009) is considered to be the main cause of cell injury, which can be induced by the dehydration from freezing, rehydration from thawing, and ice crystal growth during the whole freeze-thaw cycles (Levitt, 1980). In research by Yamazaki *et al*. (2008), the freezing tolerance to mechanical stress in *Arabidopsis thaliana* is enhanced after cold acclimation by the extracellular calcium at both the cellular and tissue levels. Calcium is likely to contribute to the structural integrity of cell walls through the formation of cross-links between carboxyl groups of adjacent polyuronide chains (Steponkus, 1984). According to our data in Figure 4.2, exogenous application of calcium is capable of improving the freezing resistance regardless of whether the cells are non-acclimated or cold acclimated. Low temperature acclimation also results in an increase of calcium levels in the cytoplasm of plants, which improves the ability of plants to better respond in the temperature-sensing mechanism which then initiates the signal transduction pathway of cold acclimation (Minorsky and Spanswick, 1989; Knight *et al*., 1996). At low temperature, the cold stimuli can trigger a series of changes including protein phosphorylation, cold acclimation-specific gene expression and freezing tolerance development (Monroy *et al*., 1993; Chinnusamy *et al*., 2007; Preston and Sandve, 2013). Collectively, these results suggest that calcium is involved in the acquisition of freezing resistance.

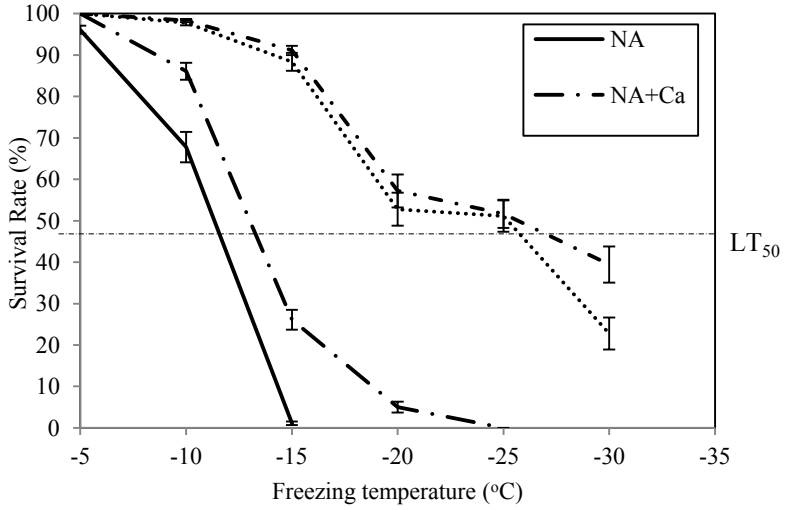


Figure 4.2 Effect of cold and calcium on freezing hardiness. The survival rate of non-acclimated cells (NA), non-acclimated cells plus $CaCl_2$ (NA+Ca), cold acclimated cells (ACC), cold acclimated cells plus calcium (ACC+Ca) plants was shown above. Error bars are means ±SD based on five separate experiments.

Although we have demonstrated increased freezing resistance of Japanese bunching onion after cold acclimation and calcium treatment, it is not known how the treated cells resist freezing damage. Our hypothesis is that the cell wall is serving as a barrier to intracellular freezing. In order to test the hypothesis, it is necessary to understand the freezing process.

4.2 Analysis of Freezing Process

Using a cryostage connected to a Nikon ECLIPSE E400 microscope and 40X objective with video recording, the temperature at which ice nucleation occurred in the extra- and intra-cellular space of tissues was monitored during freezing. Subsequently, cell viability after thawing to 24^{o}C was also observed (Table 4.1, 36 direct observations, see Appendix D for the complete set of data).

To study the ice-nucleation process, non-acclimated (plus- and minus-calcium) and two-week cold acclimated epidermal layers were incubated in the presence and absence of an extracellular nucleating agent, AgI. The application of exogenous ice nucleators plays an effective and vital role in triggering ice generation at a relative high temperature (-2 to -5^{o}C) (Table 4.1). In our system, there was no spontaneous ice nucleation of the intracellular space in any of the > 40 samples tested across all treatments (Table 4.1). Thus, intracellular ice nucleators either do not exist or are inactive in the epidermal layer of onion. When intracellular nucleation did occur, this process was due to ice propagation from the extracellular space (apoplast) into the intracellular space (see the video in Appendix C). In the absence of artificial extracellular nucleation, freezing of non-acclimated cells (NA) spontaneously initiated in the extracellular space around -8^{o}C and was immediately followed by intracellular ice nucleation with ice penetration through the apoplast into the intracellular space (Table 4.1). In the NA cells, there was no significant difference between the nucleation temperature of the extracellular and intracellular space. However, when artificial extracellular nucleation was induced around -4^{o}C in NA+Ca cells, the temperature at which subsequent intracellular ice nucleation occurred (caused by propagation of ice across the apoplast into the cell) was delayed to around -8^{o}C (Table 4.1). Without artificial extracellular nucleation, calcium treatment added to NA cells did not delay ice propagation from the extracellular to the intracellular space. However, the combination of calcium treatment and artificial extracellular nucleation (NA + Ca) (+) almost completely prevented ice propagation across the apoplast, and prevented intracellular ice nucleation. In this

case, the intracellular space of NA samples supercooled and the cells were alive to at least -10°C as determined by re-warming the cryostage to +24°C, and observing in protoplast streaming.

Table 4.1 Summary of extracellular and intracellular freezing temperature in NA, ACC and NA+Ca treated plants

Treatment	AgI extracellular nucleating agent	Mean extracellular nucleation temperature (°C)	Mean intracellular nucleation temperature (°C)
NA	-	-8.2±1.1	-8.3±1.1
NA	+	-4.1±2.2	-8.4±1.2
ACC	-	-10.1±2.3	-15.1±4.8
ACC	+	-3.2±0.9	< -21.8±2.4
NA+Ca	-	-9.6±1.8	-9.6±1.7
NA+Ca	+	-3.1±0.8	< -20.7±3.3

NA: non-acclimated cells, ACC: cold acclimated cells,
NA+Ca: non-acclimated cells plus $CaCl_2$
- no extracellular AgI nucleating agent added
+ extracellular AgI nucleating agent added
Data represents a total of 36 separate experimental observations.

The initial nucleation and growth of ice crystals in the extracellular space drives cellular water to the extracellular space from the protoplasm due to the low external cell water potential caused by the extracellular freezing, resulting in cell dehydration (Toivio-Kinnucan and Stushnoff, 1981). Once ice propagates across the cell wall and plasma membrane, intracellular freezing occurs, which is fatal to all biological cells (Fujikawa *et al.*, 1999). In nature, plant tissues can avoid intracellular freezing by tolerating freeze desiccation (Burke *et al*., 1976). In our study, the combination of calcium with artificial extracellular ice nucleation changed the NA freezing process into extracellular freeze dehydration stress similar to the freezing process of highly acclimated samples. Generally, the freezing processes involve seven types (Gusta *et al*., 2009) which were summarized in the Literature Review part of this thesis. One of the mechanisms involves supercooling in which the cells are not frozen below the freezing point. Survival of supercooled tissues at the temperature of liquid nitrogen (-196°C) has been observed in some temperate perennial plants (Burke *et al*., 1976; Lipp *et al*., 1994).

Without artificial nucleation, the freezing temperature of the intracellular space was only decreased by about 5^{o}C (-10^{o}C extracellular freezing temperature to -15^{o}C intracellular freezing temperature) and the intracellular freezing temperature was not significantly different than the extracellular freezing temperature (Table 4.1). Similar to the NA + Ca and artificial extracellular nucleation, the ACC treatment in combination with artificial extracellular nucleation (at -3 to -4^{o}C) resulted in almost complete prevention of ice propagation across the cell wall and plasma membrane. Virtually no intracellular ice nucleation was observed. Therefore, a barrier to avoid the growth of ice crystals must exist in all types of freezing processes under either avoidance or tolerance strategies. This barrier is able to not only prevent ice propagation into a cell or from stems into flowers and fruits but also decrease the loss of intracellular water because of extracellular freezing (George and Burke, 1977; Ashworth and Abeles, 1984; Wisniewski and Davis, 1995; Workmaster *et al*., 1999). Ice crystals in rhododendron and *Holcus lanatus* leaves are formed first on the leaf surface and the growth of ice is delayed through this area (Wisniewski and Fuller, 1999; Pearce and Fuller, 2001). Ice is formed and propagated from the cortex to the xylem in peach stem (Wisniewski *et al*., 1997). The thick cuticles and xylem ray parenchyma cells play critical roles in preventing ice propagation into the cells as the barrier.

The epi-cuticular layer (Figure 4.3) is the first physical barrier to prevent ice propagation into the cells of the whole plant. The epi-cuticular layer is attached to the epidermal cells through the middle lamella and cell wall. The properties such as porosity and permeability of the cell wall are thus essential factors affecting extracellular ice formation. Once ice crystals are generated in the extracellular space, the risk of cell dehydration increases and finally cell collapse occurs (Rajashekar and Burke, 1996). With cold acclimation, the thickness of leaf cell walls increases, protecting the cells against intracellular freezing (Griffith and Brown, 1982; Stefanowska *et al*., 1999). Under abscisic acid-induced freezing tolerance, cell wall thickness is also significantly increased and the majority of externally added ^{14}C-sucrose incorporates into this insoluble fraction over time, paralleling the enhancement of freezing tolerance (Tanino *et al.*, 1991). In our study, we provide direct visual evidence that intracellular freezing was delayed or did not occur in the cold acclimated onions, which may be related to compositional and structural changes of the onion cell walls. To our knowledge, this is the first time that such visual observation of ice propagation from the extracellular space to nucleate the intracellular space has been recorded.

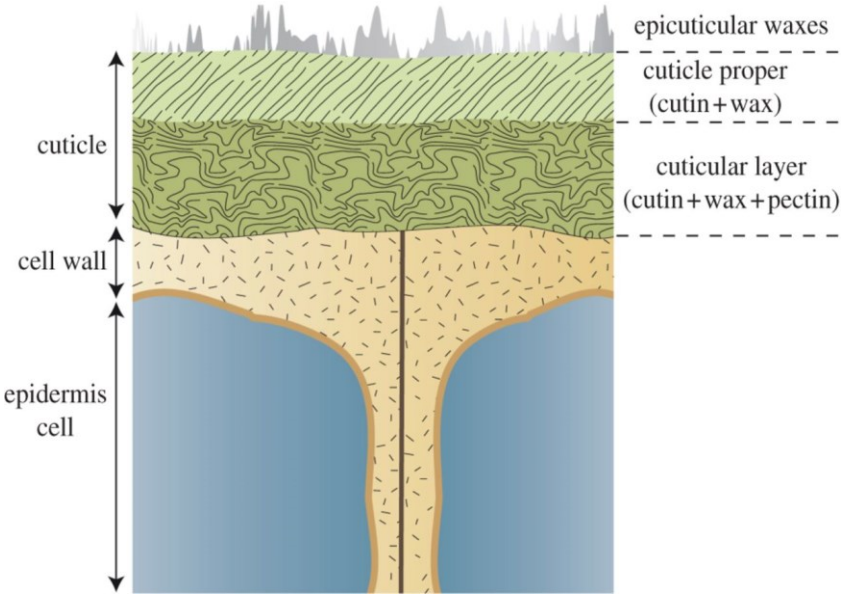


Figure 4.3 Organization of the plant cuticle (Kourounioti *et al*., 2013). Epidermal cells produce the cuticle, a polymer matrix known to contain cutin, waxes and sometimes polysaccharides (pectin) of the cell wall.

As mentioned, there was no spontaneous intracellular freezing prior to the extracellular freezing in any of these NA and ACC samples, no matter how low the temperature of supercooling. Thus, there do not appear to be active ice nucleators which initiate intracellular freezing at a high (less negative) temperature. Without artificial nucleation, extracellular freezing initiated at relatively low temperatures (average of -9^{o}C) in all treatments and thereafter tissues supercooled to relatively low temperatures before freezing. In instances when supercooling occurred (no freezing) and tissues were re-warmed from this supercooled state, cells were observed to be viable through active protoplasmic streaming (see Appendix D). However, once extracellular freezing spontaneously initiated at low temperatures in the supercooled state, intracellular freezing immediately followed in all cases (19 out of 19 samples, NA, NA+Ca and ACC) with the average subsequent intracellular nucleation temperature of –11^{o}C. Not surprisingly, all these cells were dead upon thawing. Acclimated tissue did not induce higher temperatures of extracellular nucleation compared to NA and thus acclimation itself did not prevent supercooling of the extracellular space. Also as expected, in the presence of the artificial nucleator in the extracellular apoplastic space, freezing was induced in this region at relatively high temperatures (average of –4^{o}C). Under these artificial extracellular nucleation conditions, subsequent intracellular freezing was either delayed (in NA samples, average of -8^{o}C) or did not

occur (NA+Ca and ACC). The combination of AgI nucleation and calcium treatment had a pronounced effect on virtually eliminating intracellular freezing even in NA samples. Olien (1961) indicated that the lower the temperature of freezing, the higher the physical force (biophysical force of ice propagating throughout the tissue), which can easily cause injury to the tissue.

Calcium is a divalent cation (Ca^{2+}) and plays various roles in plant growth and development, such as a macronutrient element in the whole plant, an intracellular secondary messenger in the cytosol, and as a structural integrity support in the membrane and cell wall (Wyn Jones and Lunt, 1967; Emanuelsson, 1984; Palta, 1990; Schiefelbein *et al*., 1992; Palta, 1996; White, 1998; White and Broadley, 2003; Hepler, 2005). Calcium exists in the cell wall by forming Ca^{2+} pectate with acidic pectin residues which are de-esterified by pectin methylesterase (PME) (Hepler, 2005; Tuteja and Mahajan, 2007). More specifically, during the formation of the cell wall, pectin releases the carboxyl groups which bind Ca^{2+} under the catalysis by PME, thus generating gel-like cross-linkages between cells. This process increases cell wall gelation and forms the numerous small pores in the cell wall. The increased gelation has the ability to maintain the integrity of the cell wall, which provides protection against freezing damage since the cell wall can be the primary physical barrier. Moreover, the combination of AgI extracellular nucleation and ACC treatment completely eliminated subsequent freezing of the intracellular space. Ice movement from the extracellular space to the intracellular space appeared to be blocked by the apoplast under these treatment combinations. Cells remained viable after extracellular nucleation to at least -10^{o}C before intracellular nucleation occured (Appendix D). However, even in the absence of intracellular freezing, with prolonged extracellular freezing, both NA+Ca and ACC cells were killed by extracellular freeze dehydration stress when observed at the limit of the cryostage (-23^{o}C). Thus, the blockage of ice alone cannot prevent injury in freezing tolerant cells. It can, however, play a significant role in relatively unhardy tissue and in those organs which survive through freezing avoidance mechanisms. Nevertheless, greater gelation of the apoplast will slow water loss during extracellular freeze dehydration stress and will also slow rehydration during thawing. These two factors may play a role in increasing freezing stress resistance in tolerant cells and should be investigated.

Although the freezing process (extra- and intra-cellular freezing) has been well documented, the process by which ice crystals pass through the cell wall is less studied. Thus,

the specific biochemical changes and regulation of these changes occurring in the apoplast to prevent ice penetration to the protoplasm after cold acclimation should be examined.

4.3 Studies of Apoplastic Permeability

Differential cell wall permeability was observed in non-acclimated and acclimated samples using a combination of fluorescent dyes and confocal microscopy. Fluorescence was observed in both the apoplast and cytoplasm of non-acclimated samples which indicated that the dextran conjugated to fluorescein penetrated into the intracellular space of non-acclimated samples up to the tonoplast (Figure 4.4a). This phenomenon was less pronounced in the acclimated sample (Figure 4.4b). Figures 4.4c and d are visible light images of the cells in Figures 4.4a and b, respectively. The pore diameters of a 70,000 and 3,000 MW dextran are about 12 and 2.6 nm, respectively (Wang and Fisher, 1994; Zhang *et al*., 2011). The smaller the pores of the cell wall, the less ice crystal penetration through the cell wall. In our case, the pore size of acclimated cells was reduced down to at least 2.6 nm since the 3,000 MW dextran could not penetrate the acclimated cell walls. In grape cells, the cell wall pore size was decreased from 3.5 nm to 2.2 nm. Similarly, cold acclimation in apple reduces the pore size from 2.9 nm to 2.2 nm after cold acclimation (Rajashekar and Lafta, 1996). Jones *et al*. (2000) measured the apoplastic permeability with fluorescent rhodamine green dyes and found the dyes in non-acclimated *Vitis vinifera* cv. Chardonnay buds could penetrate through the primordia because of the permeable bud axis. However, it did not occur in the acclimated buds since a permeability barrier developed that prevented dye propagation. This is consistent with the decreased permeability of the acclimated onion cells, which supports the role of the cell wall in the epidermal onion layer as a barrier.

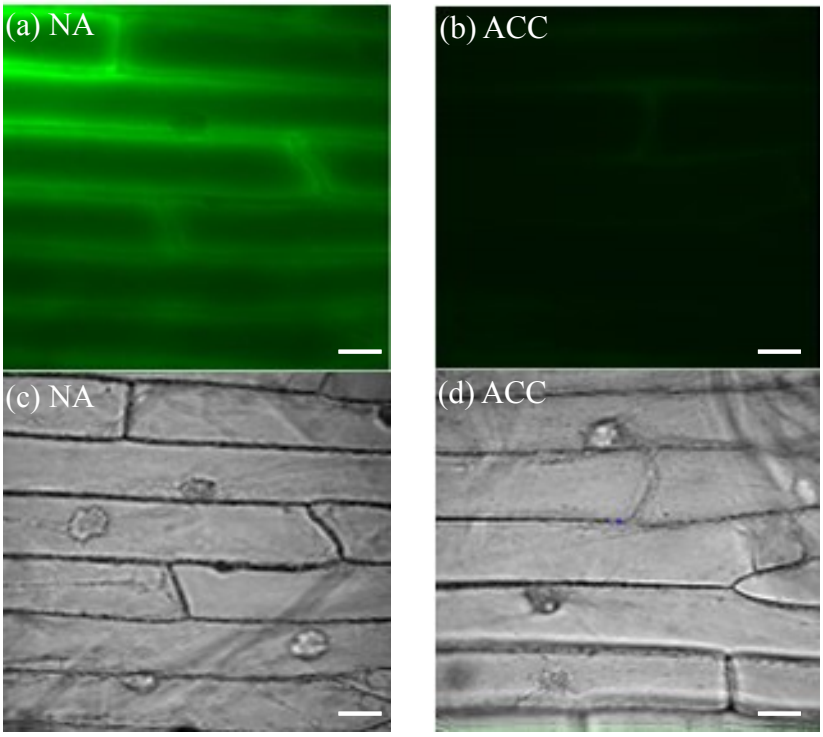


Figure 4.4 The impact of acclimation on apoplast permeability. The penetration of fluorescein conjugated with dextran (3,000 and 70,000 MW) between non-acclimated (NA) and acclimated (ACC) was measured using a confocal microscope (50X). a: non-acclimated sample; b: 2 week acclimated sample; c: non-acclimated sample with white light; d: 2 week acclimated sample with white light. Scale bar = 50 μm.

In order to evaluate how small the pore size might be, we used the smallest known diameter fluorescent molecule available, fluorescein without conjugation which has an effective radius of 6.5 Å (equal to 1.3 nm in diameter) (Lawrence *et al*., 1994). The diffusion rates of fluorescein across the cell wall to the intracellular space between NA and ACC were measured based on the brightness of fluorescence with a confocal laser scanning microscope (LSM) and NIS-Elements software. According to the video from the LSM (Video in the Appendix E), the fluorescein penetrated into the NA cells almost immediately after application to the extracellular space. By contrast, the fluorescein in the ACC samples was either markedly delayed or did not penetrate at all the cells. In Figure 4.5, the calculated mean diffusion rate in NA was 28.95 pixels per second, which was 70 times faster than in ACC cells (0.41 pixels per second).

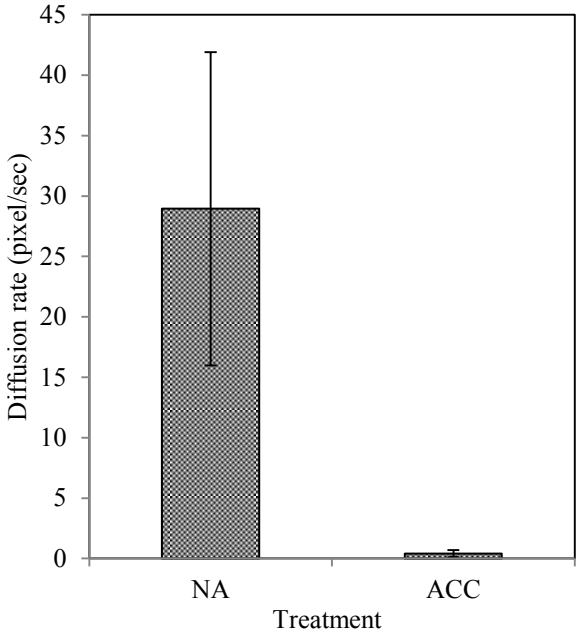


Figure 4.5 The diffusion rate of fluorescein in non-acclimated (NA) and cold acclimated (ACC) cells. Error bars are means of 5 replications ±SD.

Fluorescein is a pH dependent dye and the fluorescence can be influenced by different pH conditions (Zhu *et al*., 2005; Lakowicz, 2006). The fluorescent density of fluorescein increases when the pH value increases from 6.9- 8.4, but it almost remain unchanged when pH value is greater than 8.4 (Zhu *et al*., 2005). Therefore, the cellular pH may also affect the measurement of diffusion rate. After cold acclimation, the onion cells may be more acidic because some pectins become hydrolysed to acidic pectin and methanol by the catalysis of PMEs. Thus, the observed fluorescence in acclimated cells should be reduced compared to non-acclimated cells. In other words, the real diffusion rate in acclimated cells may be greater than the calculated value above. According to the study from Carpita *et al*. (1979), the cell wall pores in living plant cells are approximately 5 nm in diameter. In un-extracted onion cell wall material, the diameter of most pores is less than 10 nm and the effective pore size *in vivo* becomes smaller because of the existence of bound water (McCann *et al*., 1990). In our cold acclimated onion cells in the majority of samples, fluorescein did not penetrate into the acclimated cells. The pore size of the onion cell walls after cold acclimation decreased, to less than 1.3 nm diameter based on the diameter of pure fluorescein. The diameter of water is around 0.27 nm at 25 or 40^{o}C (Schatzberg, 1967), while the diameter of supercooled water is only about 9 μm at -46^{o}C (Sellberg *et al*., 2014). In non-acclimated onion cells, water can freely flow in and out of the cells. Conversely,

this phenomenon may be restricted in the cold acclimated cells as a consequence of smaller pore size of the cell wall. Ashworth and Abeles (1984) also indicated water freezes at very low temperatures when contained within small pores (less than 100 nm). According to the melting point curve, water was assumed to freeze at -15 to -25 ^{o}C when the pore size of capillaries is 4 nm in diameter (Ashworth and Abeles, 1984). The diameter of pores in non-acclimated onion cell walls is between 0-10 nm, so the freezing temperature was predicted to be slightly higher than -15^{o}C. In contrast, the freezing temperature in cold acclimated onion cells is expected to be much less than -25^{o}C since the pore size in our acclimated onion cells is less than 1.3 nm diameter (according to fluorescein tests). These smaller wall pores may effectively reduce the freezing point of intracellular water and prevent the growth of ice crystals into the cells. The cell wall permeability is affected by the cell wall porosity which is regulated by the pectin gelation. Pectin gelation can be modulated by the degree of pectin methyl-esterification and the activity of pectin methylesterases (PMEs). Therefore, both the degree of pectin methyl-esterification and the activity of pectin methylesterases (PMEs) in Japanese bunching onions were measured in the following experiments.

4.4 Analysis of The Degree of Pectin Methyl-esterification

To study the degree of pectin methyl esterification, the monoclonal antibodies JIM 5 and JIM 7 were used (Figure 4.6). Monoclonal antibody JIM5 binds to partially methyl-esterified epitopes of homogalacturonic acid (HG) and can also bind to un-esterified HG; and JIM7 binds to partially methyl-esterified epitopes of HG but does not bind to un-esterified HG (Knox *et al*., 1990; Clausen *et al*., 2003; Verhertbruggen *et al*., 2009). The fluorescence of JIM5 HG epitopes in ACC ligule cells (Figure 4.6b) was distinctly stronger than in NA control cells (Figure 4.6a). With JIM7 application, there was a slight reduction in fluorescence intensity in the ACC treatments compared to NA (Figure 4.6c and d). These data were initially obtained from Norwegian University of Life Sciences Imaging Lab, Ås, Norway and later confirmed at the University of Saskatchewan where the same experiment was repeated in NA and ACC Japanese bunching onion epidermal layers with cross sections. A stronger signal of JIM5 and weaker signal of JIM7 in acclimated cells from epidermal layer was noted when compared to the non-acclimated cells (see APPENDIX G). The stronger signal of JIM5 in the cold acclimated onions indicates the un-esterified pectins increased after cold acclimation, which may help to form more gelation through calcium cross-linkage, thereby decreasing the cell wall permeability and

preventing the growth of extracellular ice crystals into the cells. By contrast, the weaker signal of JIM7 showed that the esterified pectins decreased after cold acclimation, which may have hydrolysed to un-esterified pectin and methanol. These results were also supported by other studies in which the labeling with JIM 5 is more noticeable than JIM 7 fluorescence in December peaches and cold-acclimated winter oil-seed rape plants, respectively (Wisniewski and Davis, 1995; Solecka *et al*., 2008).

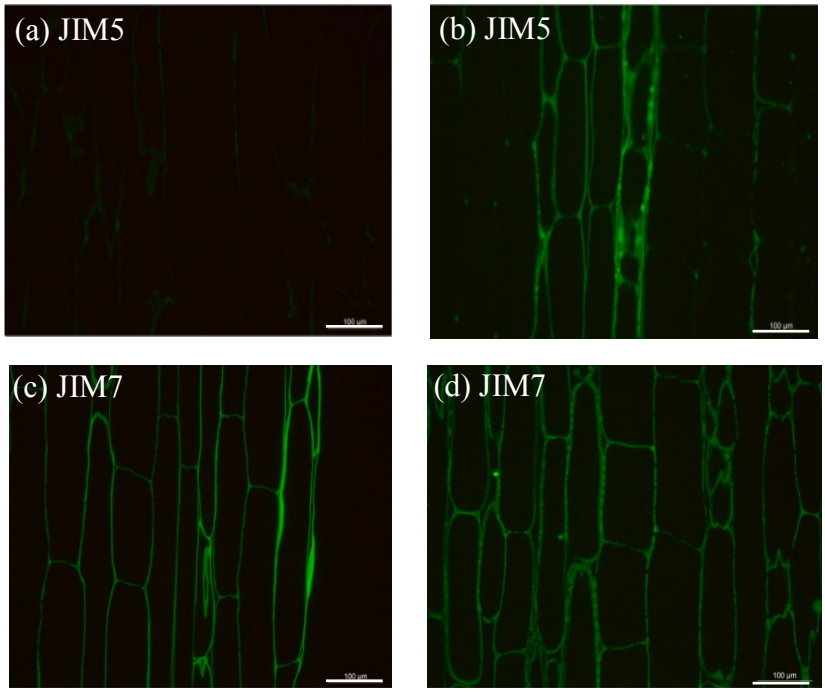


Figure 4.6 Immunohistochemical studies of pectin composition in non-acclimated (NA) and acclimated (ACC) epidermal layer cells with monoclonal antibodies JIM 5and JIM 7. a: non-acclimated cells with JIM 5, b: cold acclimated cells with JIM 5, c: non-acclimated cells with JIM7, d: cold acclimated cells with JIM7. Scale bar = 100 μm.

The apoplastic space is composed of the region outside of the plasma membrane which includes the cell wall and middle lamella. It plays significant roles in the interaction between the plant and the environment. The cell wall provides a primary natural barrier to prevent damage from the hostile environment to the plant. As one of the major cell wall components in Type I cell walls, pectin forms a hydrated cross-linked three-dimensional network in the matrix of primary plant cell walls and interlocks with hemicellulose to help to bind adjacent cells together (Carpita and Gibeaut, 1993; Voragen *et al*., 2009). It also regulates the porosity and permeability of the cell wall (Baron-Epel *et al*., 1988; Wolf *et al*., 2009; Peaucelle *et al*., 2012) and provides a defense against pathogens such as powdery mildew, in which pectin accumulation is released by

the hydrolytic enzymes (pectate lyase), eliciting the defense response to mildew (Vogel *et al*., 2002; Vorwerk *et al*., 2004). Pectins also appear to be involved in drought stress in which cell walls in the albedo of immature lemon fruitlets are thickened and its high water-binding capacity is maintained by the high content of pectin (Schröder *et al*., 2004). Under freezing stress, resistance was improved by modifications in the degree of pectin methyl esterification (Wisniewski and Davis, 1995; Solecka *et al*., 2008). In Wisniewski and Davis's study (1995), the labelling with JIM7 recognizing highly esterified epitopes of pectin was distributed in the whole pit membrane, primary cell walls of xylem and floral bud tissues of peach, while the labelling with JIM5 recognizing un-esterified epitopes of pectin was located outside of the pit membrane in xylem tissues and in middle lamellae of floral buds. These results revealed the distribution and types of pectin in both xylem and floral bud tissues of peach. It has been known that deep supercooling occurs in the xylem tissues and floral buds in peach. Combining the results above, pectin in the xylem tissues and floral buds greatly contributes to deep supercooling, avoiding freezing damage. Meanwhile, JIM5 signal in December peaches was stronger than in July/August samples, indicating there were more un-methylesterified pectins in December peaches which could survive in the winter. Pectins are involved in enabling freezing avoidance to occur in xylem ray parenchyma of deep supercooling trees through ice blockage by the xylem pits (Ashworth and Abeles, 1984; Wisniewski *et al.*, 1991a). However, the study from Baldwin *et al*. (2014) indicated higher immunolabeling with JIM7 in the frost-tolerant pea genotype 'Champagne' than the frost-sensitive pea genotype 'Terese', which indicates an increase in the degree of methyl-esterification of pectins after cold acclimation.

4.5 Cell Wall Compositional Analysis

4.5.1 Determination of Cell Wall Quantity in Ligule Tissue

The relative crude cell wall preparations of the epidermal layer, non-epidermal layer and the whole sheath (composed of both epidermal layer and non-epidermal layers) during cold acclimation were analyzed. The epidermal layer contained significantly higher relative cell wall content (insoluble fractions), occupying up to 22.6 % of fresh sample weight after 14 days cold acclimation compared with NA onion epidermal peels (Figure 4.7). The relative cell wall content in the non-epidermal layers and the whole sheath increased slightly during cold acclimation. It has been shown that the thickness of the cell wall increases significantly in the hardened winter

rye (Griffith *et al*., 1985) and in the 7-days ABA-acclimated bromegrass cells (Tanino *et al*., 1991). The cell wall content in winter oil-seed rape leaves also increases after cold hardiness (Kubacka-Zębalska and Kacperska 1999; Solecka *et al.* 2008), which is consistent with the result from the ACC onion cell walls. The research from Stefanowska *et al*. (1999) illustrated increased cell wall content was related to the increased thickness of cell wall in epidermis cells. The changes of the cell wall structure were dependent on cold hardiness and may have contributed to the protection from freezing-induced damage since these modifications increase leaf tensile stiffness which avoids cell volume collapse and cell wall deformation in response to extracellular freezing (Pearce, 1988; Fujikawa *et al*., 1999; Stefanowska *et al*., 1999; Jarvis and McCann, 2000).

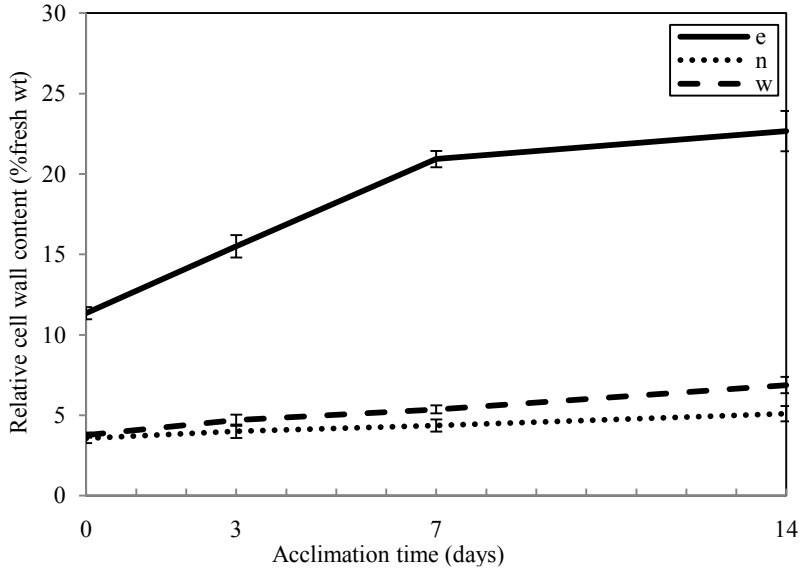


Figure 4.7 The relative content of onion crude cell wall mass during cold acclimation. Values are expressed by the percentage ratio of the crude cell wall fresh weight of respective tissue. e: epidermal layer, n: non-epidermal layer, w: whole sheath (e+n). Error bars are±SD of seven separate experiments.

4.5.2 Cell Wall Carbohydrate Analysis of Ligules

The individual cell wall components in the epidermal layer were quantified by HPLC (GC/MS) analysis and partitioned into TFA insoluble (trifluoroacetic acid) and water soluble fractions. As expected, the insoluble TFA fraction contained a higher concentration of cell wall components than the water soluble fraction. The cell wall polysaccharides of onion contain fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid (Ng *et al*., 1998). The major components within the onion epidermal layers were

galactose and galacturonic acid, representing 28.5% and 35.5% of the total cell wall fraction, respectively (Figure 4.8). Galacturonic acid (GalA) including methylated and un-methylated residues is the main component of pectin, representing around 70% of homogalacturonan (Mohnen, 2008). Carbohydrate composition was similar between NA and ACC except for galacturonic acid in which the ACC samples contained slightly higher about 27% galacturonic acids than non-acclimated onion cells in the insoluble fraction. Pectic polysaccharides are the major group of onion cell wall constituents, occupying 42.2% of the cell wall (Mankarios *et al.*, 1980).The higher content of GalA in the cold acclimated onion cells implies there was more pectin in acclimated onion cells than in the non-acclimated onions. This is consistent with the studies of pea (Weiser *et al*., 1990), winter oil-seed rape plants (Kubacka-Zębalska and Kacperska, 1999; Stefanowska *et al*., 1999; Solecka *et al*., 2008) and the tolerant pea cultivar 'Champagne' (Baldwin *et al*., 2014) during the period of cold acclimation.

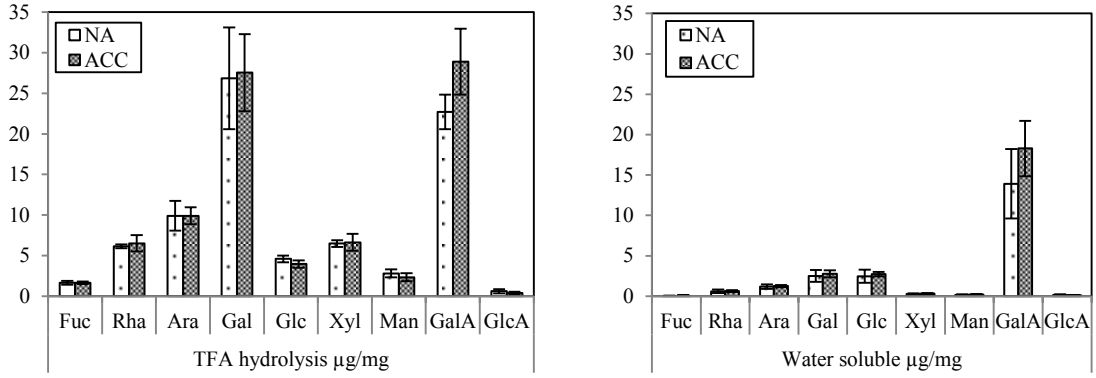


Figure 4.8 Cell wall components of the epidermal layer. Data were obtained by analysis of GC/MS NA and ACC of epidermal ligule cells. The cell wall components include trifluoroacetic acid (TFA) fraction (insoluble) and water soluble fraction. NA: non-acclimated onion cells, ACC: cold acclimated cells. Error bars are ± SD of two separate experiments. Fuc: fucose; Rha: rhamnose; Ara: arabinose; Gal: galactose; Glc: glucose; Xyl: xylose; Man: mannose: GalA: galacturonic acid; GlcA: gluconic acid.

4.6 PME Activity in Epidermal Tissues of Ligules

4.6.1 Determination of Total Cell Wall Protein Content

The relative quantity (% cell wall protein/total crude cell wall) of total cell wall protein in the epidermal layer was two to three times greater in the epidermal layer than in the non-epidermal layer in both NA and ACC tissue (Figure 4.9). Specifically, the total cell wall protein occupied 4% of the total crude cell wall after 14 days cold acclimation, which was significantly

greater than the non-epidermal layer and the whole sheath (Figure 4.9). However, the rate of cell wall protein increase appeared to be similar between tissues over 14 days of acclimation (Figure 4.9). The plant cell wall protein profile includes in decreasing order of concentration: antifreeze proteins (Duman and Olsen, 1993; Griffith *et al*., 2005; Yu *et al*., 2010), arabinogalactan proteins (AGPs) (Gong *et al*., 2011), expansins (Noh *et al*., 2009), glycine-rich proteins (GRPs) (Ferullo *et al.*, 1997), xyloglucan endotransglucosylase/hydrolases (XTHs) (Yun *et al*., 2007), extensin (Weiser *et al*., 1990), and related cell wall enzymes like pectin methylesterases (Kubacka-Zębalska and Kacperska, 1999; Solecka *et al*., 2008; Baldwin *et al*., 2014). This profile may change during cold acclimation and these proteins may up- (AGP, expansins) or down-regulate (extension and XTHs) gene expression, modifying the mechanical properties of the cell wall such as mediating cell-wall loosening by expansins (Li *et al*. 2003) and determining the cell wall plasticity by XTH (Chanliaud *et al*., 2002), thereby potentially improving the freezing tolerance of plants (Yun *et al*., 2007; Noh *et al*., 2009; Shi et al., 2014; Gall *et al*., 2015).

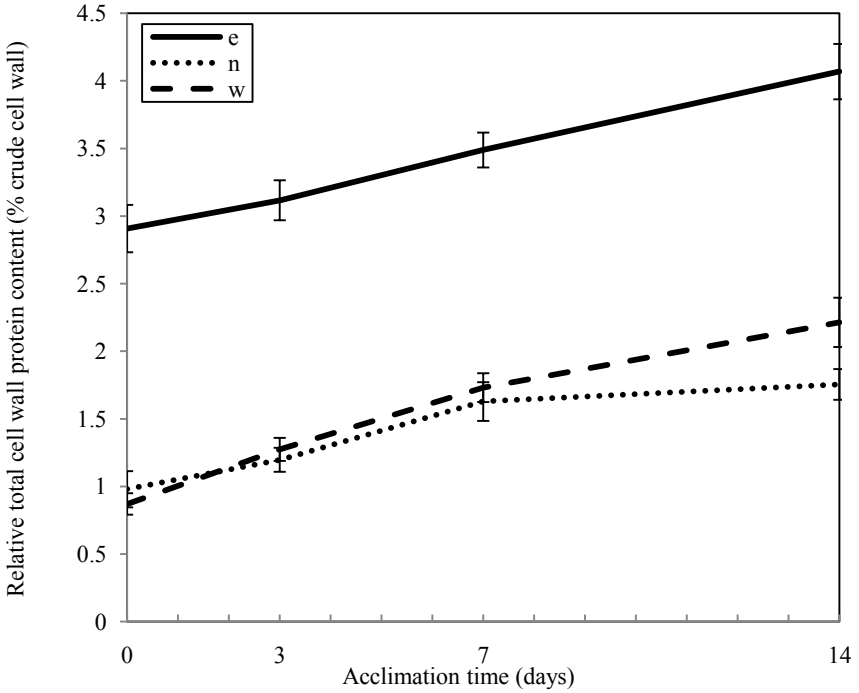


Figure 4.9 Relative total cell wall protein content during cold acclimation. Values are expressed by the percentage ratio of the total cell wall protein content to the crude cell wall FW. e: epidermal layer, n: non-epidermal layer part, w: whole sheath. Error bars are ± SD of seven separate experiments.

4.6.2 PME Activity Analyses

Pectin methylesterases (PMEs, EC 3.1.1.11), a cell wall-associated enzyme in the apoplast, catalyses the hydrolysis of the methyl ester group of pectin (mainly homogalacturonan) to release acidic pectin and methanol; the carboxyl acid residues from this reaction can be cross-linked by calcium, forming calcium-pectate gels, which can increase the stiffness of the cell wall (Jarvis, 1984; Bosch, 2005). PME activity (expressed as nEq H^+ mg^{-1} protein min^{-1}) in the cell wall was separated into the soluble and cell wall-bound enzyme fractions (Figure 4.10). Overall, the PME specific activity in the soluble fraction followed a decreasing tendency over the 14 day ACC period (Figure 4.10a). By contrast, PME had an increasing tendency in the cell wall-bound fraction following the acclimation period (Figure 4.10b). The relative PME enzyme activity in the epidermal layer was significantly higher than the non-epidermal layer and the whole sheath. Specifically, after two weeks of cold acclimation, the relative PME activity in the epidermal layer (expressed as a function of each tissue's respective fresh weight) was more than two times that of the non-epidermal layer (Figure 4.10c).

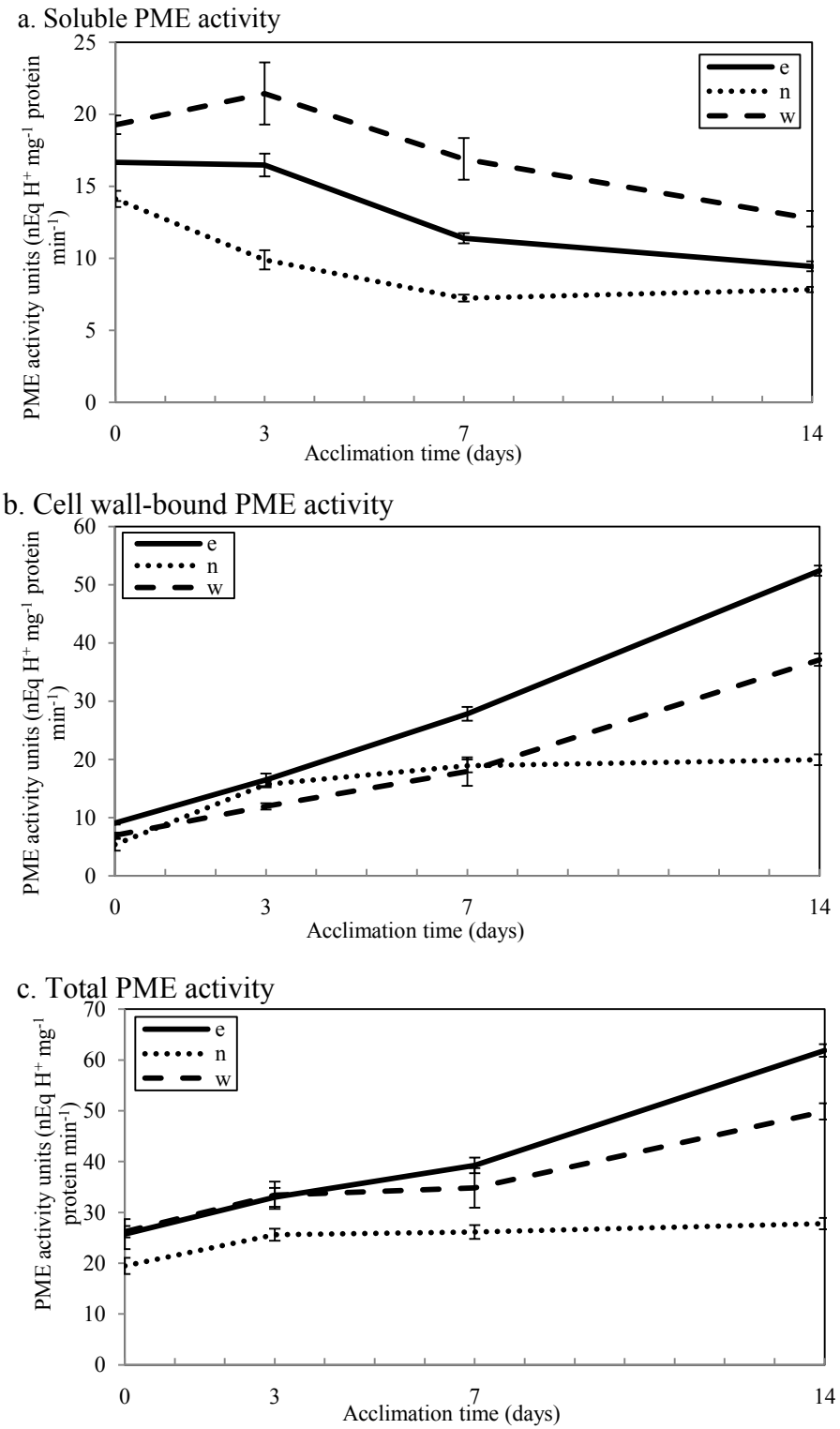


Figure 4.10 Relative PME specific activity during cold acclimation. The PME activity unit was expressed by nEq H^+ mg^{-1}protein min^{-1}. a: soluble PME activity, b: wall-bound PME activity, c: total PME activity (including soluble and wall-bound PME activity), e: epidermal layer, n: non-epidermal layer part, w: whole sheath (e+n). Error bars are means of five replications ±SD.

There are two main types of primary cell wall among species of angiosperms: Type I cell walls from eudicots and some monocots, and Type II cell walls from the Poaceae. In Type I cell walls, the network of cellulose microfibrils is tethered with xyloglucan polymers and embedded in a gel of pectic polysaccharides through the cross-linking with calcium. By contrast in the Type II cell walls, the microfibrils are interlaced with glucuronoarabinoxylans rather than xyloglucans, and there is rarely pectin (Carpita and Gibeaut, 1993). The cell walls of onions belong to the Type I cell wall which contain abundant pectic matrix (Carpita and Gibeaut, 1993). Pectins impart various physical characters to the cell walls, such as density, wall porosity, the distribution of wall enzymes and proteins, and cell-to-cell adhesions (Albert *et al*., 2002). The methylester groups of cell wall pectins are hydrolysed to acidic pectin and methanol through de-methylesterification by PME (O'Neil *et al*., 1990; Pelloux *et al*., 2007). The acidic pectins with free carboxyl groups are capable of forming cross-links with calcium, increasing the gelation of pectin, thereby reducing the pore size and permeability of cell walls. The ability of cell walls to resist extracellular ice nucleation into the cells may be improved through the modification of the cell wall structure. There is a negative correlation between homogalacturonan (HG) methyl esterification and PME activity in chilling-injured peach fruit after storage at 5^{o}C for 1 to 4 weeks, in that increased PME activity reduces the accumulation of methyl esterified HG, which increases cold storage of peach (Brummell *et al*., 2004). Un-methylesterified HG can form a gel through cross-linkage with calcium, which in turn may regulate the freezing resistance by enhancing the formation of gelation and cell wall porosity (Willats *et al.*, 2001), thereby potentially decreasing the risk of ice penetration into the cells. PME activity in oil seed rape plants increases after cold acclimation (Solecka *et al*., 2008), which is consistent with our result in cold acclimated onions. Cell wall rigidity is also affected by de-methylesterification of HG (Willats *et al*., 2001; Solecka *et al*., 2008). The plant cell walls play a critical role in withstanding the hydraulic turgor pressure of the cells, which combine with wall construction to contribute to cell wall rigidity (Lodish *et al*., 2000). During freezing stress, extracellular ice nucleation induces intracellular dehydration stress which lowers internal water potential and leads to the decrease in the turgor pressure. Increased cell wall rigidity accompanied by solute accumulation helps to maintain cell volume at low water potentials during drought stress (Clifford *et al*., 1998). Thus, cell wall rigidity increases in response to lowering water potential under intracellular dehydration stress. Ha *et al*. (1997) measured the cell wall rigidity in dry and hydrated onions with solid-state

nuclear magnetic resonance relaxation and found that both pectic matrix and microfibrils provide mechanical properties and rigidity to the cell walls. In addition, the transfer from the esterified pectins in the apical pollen tube to un-esterified pectins in the distal pollen tube was thought to be associated with the increase in the cell wall rigidity (Parre and Geitmann, 2005). The overall cell wall rigidity appears to result from the un-methylated pectins (Fenwick *et al*., 1997). After cold acclimation, the amount of un-methylated pectins increased (Fig.4.6) mainly due to the higher PME activity comparing the non-acclimated cell walls. More un-methylated pectins mean more rigid cell walls. Therefore, the rigidity of the cell wall is improved by the increasing PME activity, and this is accompanied by the ability of cell wall to resist freezing damage.

As compared with common onions (*A.cepa*) and green onions (*A. chinense*), Japanese bunching onion (*A. fistulosum*) had higher PME activity in soluble, bound and total fractions (Figure 4.11). *A. cepa* is very sensitive to low temperature and has a low freezing resistance with -11^{o}C of LT_{50} value (Palta *et al*., 1977), while LT_{50} in *A. fistulosum* is -27^{o}C. The high PME activity in *A. fistulosum* may explain why Japanese bunching onion has the highest freezing resistance capability among these three species.

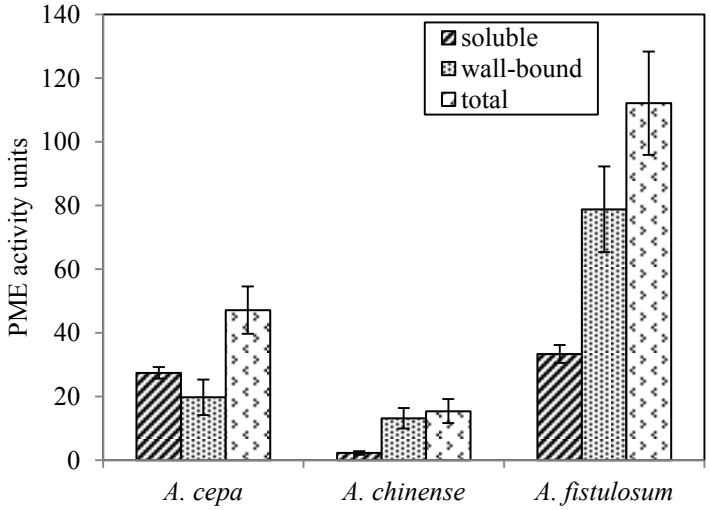


Figure 4.11 The PME activity in the whole sheath (including epidermal layer and non-epidermal layer) of onion species: Common onion (*A.cepa*), green onion (*A. chinense*) and Japanese bunching onion (*A. fistulosum*) were analyzed. Error bars are means of four separate experiments ±SD.

PME also plays a vital role in dormancy breakage (Ren and Kermode, 2000), seed germination (Ren and Kermode, 2000; Müller *et al*., 2013), elongation growth (Bordenave and Goldberg, 1993; Peaucelle *et al*., 2012), plant development (Wolf *et al*., 2009), fruit ripening

(Kagan-Zur *et al*., 1995; Draye and van Cutsem, 2008), pathogen defense (Lionetti *et al*., 2007; Volpi *et al.*, 2011). PME activity affects the plant-pathogens interactions (Micheli, 2001; Lionetti *et al*., 2012), but the way in which PME acts on plant pathogen resistance is opposite from freezing stress. As one of the cell wall degrading enzymes (CWDEs), PME affects the degree of pectin methyl esterification. A series of studies have revealed that the resistance to *Pectobacterium carotovorum* in potato cultivars (McMillan *et al*., 1993; Marty *et al*., 1997), *Colletotrichum lindemuthianum* in resistant bean cultivars (Boudart *et al*., 1998) and *Ralstonia solanacearum* in tomato genotypes (Wydra and Berl, 2006) are correlated with higher degree of pectin methyl esterification. Therefore, in contrast to the function of PME activity in freezing stress, the reduced susceptibility of plants to the various pathogens is dependent on the low PME activity and high degree of pectin methyl esterification (Lionetti *et al*., 2007; Lionetti *et al*., 2012).

In our study, the Japanese bunching onion was used as a model system to better understand the mechanisms of intracellular freezing stress avoidance based on cell wall alterations. The LT_{50} decreased from -12^{o}C to -15^{o}C in one-week acclimated samples and significantly lowered to -27^{o}C after two weeks of cold acclimation. Freezing resistance of Japanese bunching onion cells increased greatly after two-week acclimation compares to one-week acclimation. Based on the measurements on the apoplast compositions during cold acclimation, the crude apoplast contents increased by 45% from non-acclimation to one-week acclimation while it slightly increased by 9% from one-week to two-week acclimation. However, the big increase of PME activity occurred from one-week (15%) to two-week (36%) acclimation, which was consistent with the increasing tendency in freezing resistance during the period of cold acclimation. This result indicated that the improving of resistance to freezing is more dependent on the efficiency of pectin methylesterase not only because of the increasing amount of the apoplast.

There is no doubt that cold acclimation plays a very important role in improving freezing resistance through modifications in morphology such as rosette shape in hardy varieties while upright shape in sensitive varieties of flax during winter (Omran *et al*., 1968), changes in physiological and molecular level such as antifreeze proteins (AFPS) accumulation in the apoplast during cold acclimation (Griffith *et al*. 2005), changes in ultrastructure such as cell wall (Alonso *et al*., 1997). In this study, we hypothesized that the alterations in cell wall composition

in response to low temperature regulates freezing avoidance through alterations in cell wall permeability. In the freezing process, ice nucleation first occurs in the extracellular space, and ice crystals may subsequently propagate through the cell walls and plasma membrane, nucleating the protoplast causing cell death. In tolerant cells, the collapse of the cell walls is due to extracellular freeze-induced cell dehydration which is related to water movement out to the extracellular space (Levitt, 1980; Rajashekar and Burke, 1996).

The cell wall porosity determines the permeability of cell wall to allow the molecules with certain molecular weight such as water and proteins to penetrate into the cells (Albersheim *et al*., 2011). The lower porosity with smaller pore size of cell wall can restrict the penetration of the big molecules like ice crystals, reducing the freezing damage to the cells (Jones *et al*., 2000). As the primary physical barrier, the mechanical properties of cell walls greatly contribute to the resistance of plants to freezing stress (Tao *et al*., 1983; Murai and Yoshida, 1998) and are affected by the amount and structure of polysaccharides in the cell walls (Taiz, 1984; Carpita and Gibeaut, 1993). Generally, the primary cell walls in most plant cells are freely permeable and allow small molecules such as water (diameter of about 0.27 nm at 25^{o}C) and some small proteins to pass through to the intracellular space. The mixture of 70,000 MW (12 nm in diameter) and 3,000 MW (2.6 nm in diameter) dextran conjugated with fluorescein in two-week cold acclimated (12/4^{o}C day/night) Japanese bunching onion was blocked out of the cells while the dye penetrated into the tonoplast region in non-acclimated cells. This is consistent with the study from Jones *et al*. (2000) who illustrated that both the dye and ice crystals cannot pass through the grape bud axes due to the permeability barrier in the axis of buds after cold acclimation. Meanwhile, they also used pectinase to remove pectin, and found that the apoplastic permeability increased and ice nucleation temperature also increased. Therefore, pectins are involved in the permeability barrier to limit the ice propagation. According to our study, cold acclimation induced an increase in the amount of galacturonic acid (GalA), the basic component of pectins (mainly homogalacturonan). In addition, un-methylated pectin increased in the cold acclimated cells as observed using immunolabeling. Cross-linkage in the plant cell walls is formed through the ionic bond combination of anionic un-methylated pectin and cationic calcium, which promotes the production of pectate gel and provides cell wall porosity (Ridley *et al*., 2001; Liu *et al*. 2009; Voragen *et al*., 2009). In this case, the cell wall has the characteristic permeability to permit the passage of molecules having suitable diameter through cell wall pores.

The survival rate in the treatment of ACC+Ca was much higher than that in ACC alone, by approximately 44%. Calcium is involved in improving the freezing resistance in response to cold acclimation. Apoplastic Ca^{2+} is the highest concentration in plant tissues, about 60-75% of the total tissue Ca^{2+} content (Demarty *et al*., 1984), playing an essential role in controlling cell integrity, cell wall cohesion and plasma membrane permeability (Hirschi, 2004). Studies have demonstrated that exogenous applications of calcium enhances tolerance to cold stress (Wang *et al*., 2009; Zhou and Guo, 2009), which is consistent with my study showing increased survival rate after calcium application regardless of non-acclimated or acclimated cells. In addition, cytoplasmic calcium levels increased rapidly in both *Arabidopsis* (Knight *et al*., 1996; Polisensky and Braam, 1996) and alfalfa (Monroy and Dhindsa, 1995) in response to low temperature, which is thought to be mainly due to an influx of calcium from extracellular stores. Cytosolic calcium has been shown to regulate the basic components of calcium signal transduction pathway, such as calmodulin (CaM), and calcium-dependent protein kinases (CDPKs), thereby improving the resistance to freezing stress since calcium serves as a second messenger in response to cold acclimation (Trewavas and Gilroy, 1991; Roberts and Harmon, 1992; Bush, 1993; Monroy *et al*., 1993). Here, the roles of calcium in the cell wall during freezing stress were addressed. High content of apoplastic Ca^{2+} provides more opportunity to bind the contiguous un-methylesterified pectin (mainly homogalacturonan) to form the 'egg-box' structures which confers the rigidity of cell wall and maintains the integrity of cell wall in order to relieve the stresses (Grant *et al*., 1973; Hamann *et al*., 2009). In turn, the un-methylesterified pectins have a high affinity with calcium ions (Goldberg *et al*., 1996). Moreover, the un-methylesterified pectin is released from the methylesterified pectin through the catalysis by pectin methylesterases (PMEs) which are a hydraulic enzyme residing in the cell wall (Micheli, 2001; Pelloux *et al*., 2007). PMEs help to form very tight intermolecular interactions (pectate gel) with Ca^{2+} (Bosch, 2005), and regulate apoplastic Ca^{2+} content in response to stresses in order to modulate the pectic network (Micheli, 2001; Pelloux *et al.*, 2007). Therefore, PMEs and apoplastic Ca^{2+} interact with each other. Guillemin *et al*. (2008) showed PME plus Ca^{2+} increased the mechanical rigidity of the cell wall structures through a vacuum-impregnation experiment. After cold acclimation, PMEs activity increased in Japanese bunching onions, resulting in more un-methylesterified pectins, tighter gel formation and smaller pore size. The pore size of the cell wall determines its permeability (Baron-Epel *et al*., 1988). In my study, I did

find cell wall permeability was reduced, according to the blockage of 3 000 MW dextran conjugated with fluorescein (2.6 nm diameter) and slower diffusion rate of pure fluorescein (1.3 nm diameter) in cold acclimated cells, thus the diameter of pore size of acclimated cell walls should be less than 1.3 nm. However, the diameter in non-acclimated cell wall pores is larger than 2.6 nm since the mixed dyes of 3,000 and 70,000 MW (12 nm in diameter) dextran conjugated with fluorescein can freely pass through non-acclimated cell walls. Furthermore, the immunolabeling test showed the stronger signal of JIM5 and the weaker signal of JIM7 appeared after cold acclimation, which further confirmed the un-methylesterified pectins increased in response to cold. Also, the increase in cell wall and pectin content may contribute to a thickened cell wall that provides a stronger barrier against ice penetration. The results from this project address my hypothesis well that alterations in cell wall compositions (pectin, especially un-methylesterified pectin) in response to low temperature improve freezing avoidance through a decrease in cell wall permeability which results from the tighter binding between un-methyesterified pectin and Ca^{2+}. Except freezing stress, it has been shown that some other stresses including drought and pathogen attacks also can be avoided through the modifications of cell wall (Micheli, 2001; Verslues *et al*., 2006; de Freitas, *et al*., 2012). Consequently, the cell wall is really a primary physical barrier to avoid environmental injuries.

5.0 CONCLUSIONS

It is well-known that intracellular freezing is lethal to all cells. As a freezing stress resistance mechanism, freezing avoidance provides plants with more potential to survive subzero temperatures without intracellular freezing. The hypothesis in this project that reduced permeability of the apoplast through pectin modification will increase freezing avoidance and freezing stress survival was supported.

In all experiments, ice nucleation in the epidermal layers initially occurred in the apoplast. We showed direct evidence that subsequent ice propagation across the cell wall leading to freezing of the intracellular space was visibly blocked by cold acclimation or $CaCl_2$ treatment. Ice blockage appears to be associated with alterations of the structure and composition of cell walls through strengthening of the ice propagation barrier. Reductions in cell wall permeability, which can affect ice spread between the extra- and intracellular space, was a consistent response to cold acclimation. The decrease in cell wall permeability after cold acclimation effectively avoided ice penetration into the intracellular space, thus avoiding freezing injury. Changes in cell wall composition were closely connected with freezing resistance as a result of cold acclimation. An increased quantity of the relative crude cell wall content is consistent with other reports showing a physical augmentation of the cell wall content and association with increased freezing stress resistance. We further examined compositional changes of the cell wall during acclimation and found increased pectic polysaccharides (galacturonic acid) and elevated demethylesterification in acclimated cells. The large increase of PME activity appeared in the two-week rather than one-week cold acclimated samples which corresponded with the large increase in freezing stress resistance at two weeks. This result illustrated that the increasing freezing resistance of Japanese bunching onion more depends on the activity of PME not only the quantity of the apoplast. Furthermore, the increase of PME activity during cold acclimation with the majority of PME localized in the epidermal layer supports the observed increase in de-methylesterified pectins. Collectively, these changes may provide more potential for pectin to cross-link with calcium to form a tighter gel which reduces the pore size of cell walls, and reduction in cell wall permeability. As a consequence, ice may be trapped outside the cells and freezing damage may be avoided.

Using *Allium fistulosum* as a system to study intracellular freezing avoidance is useful

since this species is easy to propagate and grow with short generation time. The species is both drought and low temperature stress resistant and the epidermal layer can be easily peeled with intact large cell size which does not autofluoresce. The results from this study indicated that freezing resistance in *Allium fistulosum* can be improved through reductions of the cell wall permeability, which is associated with pectin dynamics in response to cold acclimation.

6.0 FUTURE WORK AND SIGNIFICANCE

Based on this study, Japanese bunching onion (*Allium fistulosum* L.) appears to be a good model system to better understand the mechanisms of intracellular freezing stress avoidance and cold acclimation-induced alterations of the cell wall structure and composition (in particular pectins) to avoid freezing injury. However, more work on this topic is needed to further determine the specific mechanism of cell wall-regulated freezing stress resistance.

Future studies can focus on detecting the cold-induced modifications of the cell wall structures, especially pectins, in *Allium fistulosum* after cold acclimation. The dynamics of cell wall structure and composition can be tested through Synchrotron Radiation-Fourier Transform Infrared (SR-FTIR) Spectroscopy in Canadian Light Source. The pore size of cell walls will be measured, which can provide more direct evidence on the decreasing permeability of cell walls during cold acclimation.

Ca^{2+} plays an essential role in controlling cell wall integrity, cell wall pore size and cohesion, and signal transduction as a second messenger in response to stress conditions (Hirschi, 2004). The apoplastic Ca^{2+} can bind the contiguous un-methylesterified pectin (mainly homogalacturonan) to form the crossing-linkage which confer the rigidity of cell wall and maintain the integrity of cell wall in order to relieve the stresses (Hamann *et al*., 2009). Consequently, the localization, quantity and pattern of calcium also can be carried out through the x-ray beamline in the Canadian Light Source, thereby better understanding how the calcium plays the role in enhancing the ability of plant to resist freezing stress. Moreover, the mechanisms on how PME activity is regulated can be tested since we have *Arabidopsis* mutants with PMEI OE (PME inhibitor overexpression), which may provide the molecular bases of the resistance to freezing stress. Therefore, the results from those tests can further confirm the roles of cell wall as a barrier in decreasing even avoiding the freezing damage.

The cell wall as a barrier may play an even wider role in stress resistance. Water loss (dehydration) is caused by extracellular freezing-induced dehydration (Yamazaki *et al*., 2009), but also desiccation and salt stress (Zhu, 2002) and postharvest desiccation (Belincontro *et al*., 2004). Ice penetration can be prevented by the decreasing cell wall permeability which is determined by the pore size of cell walls. There is some evidence that pectins are involved in the permeability barrier to limit the ice propagation (Jones *et al*., 2000) because the acidic pectins

are capable of forming the pectate gel with Ca^{2+}. The acidic pectins which are un-methylated are the products of the hydrolysis of methylated pectins under the catalysis of pectin methylesterase (PME), so the pectin gelation is directly regulated by the PME activity. Therefore, PME activity in the freezing resistance is important since PME controls the cell wall permeability that can reduce and even avoid injury from ice penetration to the internal cells.

We found PME activity was highest in the epidermal layer and increased during cold acclimation in both the epidermal layer and underlying non-epidermal layers. A similar result was also found in the Solecka *et al*.'s research (2008) in which tissue resistance to blackleg fungus may have been related to the modifications of leaf cell wall structure and composition (especially pectin) in response to low temperature. PME gene expression also increases upon cold-treatment of *Arabidopsis* pollen (Lee, 2003).

PME contributes to the resistance of plants to pathogens and the plants having low PME activity are more resistant to various pathogens (McMillan *et al*., 1993; Marty *et al*., 1997; Boudart *et al*., 1998; Wydra and Berl, 2006; Lionetti *et al*., 2007; Lionetti *et al*., 2012). Consequently, research on PME in response to freezing and pathogens should be expanded to different plant species exposed to various abiotic and biotic stresses. Based on desired PME property, breeders can select for freezing avoidance (high PME activity) or pathogen resistance (low PME activity). As a consequence, the study on PME may provide a possibility to directly select the freezing- or pathogen- resistant species. This possibility also can be examined in other stress conditions such as drought and salt stress.

In addition, we need to understand any mechanisms to resist the environmental stress not only depend on one aspect such as cell wall (mainly pectin) but also rely on other factors like plasma membrane. Actually, most of researches on freezing resistance focus on the roles of plasms membrane. Understanding the whole cellular functions in the process of freezing resistance is very critical in a comprehensive way.

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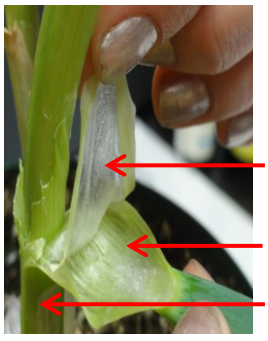
APPENDIX A: Growth and morphology of *Allium fistulosum* L.

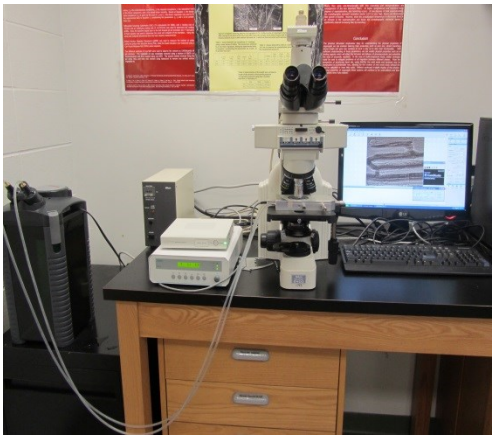
Allium fistulosum L. growing in soilless mix in the College of Agriculture and Bioresources greenhouse at University of Saskatchewan

The leaf number sequence of *Allium fistulosum* L.



The single epidermal layer of *Allium fistulosum* L.

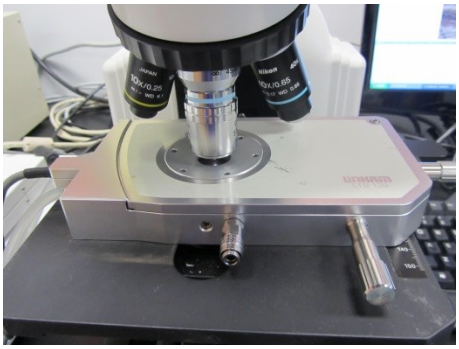
APPENDIX B: The cryostage microscope equipment



The whole view of cryostage microscope

Upper: Nikon camera attachment
Lower: Linkam PE94 controller

Heat sink unit



Linkam freezing stage, LTS 120

APPENDIX C: Protoplastic streaming and ice penetration in the cells


media1.wmv Live cells with protoplasmic streaming


media2.wmv Dead cells without protoplasmic streaming


media3.wmv Ice penetration into non-acclimated cells

APPENDIX D: Analysis of freezing behavior in treated epidermal layer cells of ligules

The cryostage behavior of NA, ACC and NA+Ca

Treatment [1]	AgI extracellular nucleating agent [2]	Freezing Temperature (^{o}C)		Viability [3]
		extracellular	intracellular	
NA	-	-7.4	no	alive
NA	-	-7.6	no	alive
NA	-	-5.3	no	alive
ACC	-	-9.4	no	alive
ACC	-	-11	no	alive
NA	-	-7.3	-7.4	dead
NA	-	-7.5	-7.5	dead
NA	-	-7.4	-7.4	dead
NA	-	-10.5	-10.6	dead
NA	-	-8.2	-8.2	dead
NA	-	-8.5	-8.5	dead
NA+Ca	-	-10.5	-10.5	dead
NA+Ca	-	-7.3	-7.4	dead
NA+Ca	-	-8.5	-8.6	dead
NA+Ca	-	-11.9	-11.9	dead
ACC	-	-10.1	-10.1	dead
ACC	-	-10.4	-17.1	dead
ACC	-	-10.2	-10.2	dead
ACC	-	-12.1	-12.1	dead
ACC	-	-10.8	down to -23 no	dead
ACC	-	-11.7	-11.7	dead
ACC	-	-7.6	-15.9	dead
ACC	-	-13.1	-13.1	dead
ACC	-	-4.10	down to -23 no	dead
NA	+	-3.1	-7.9	dead

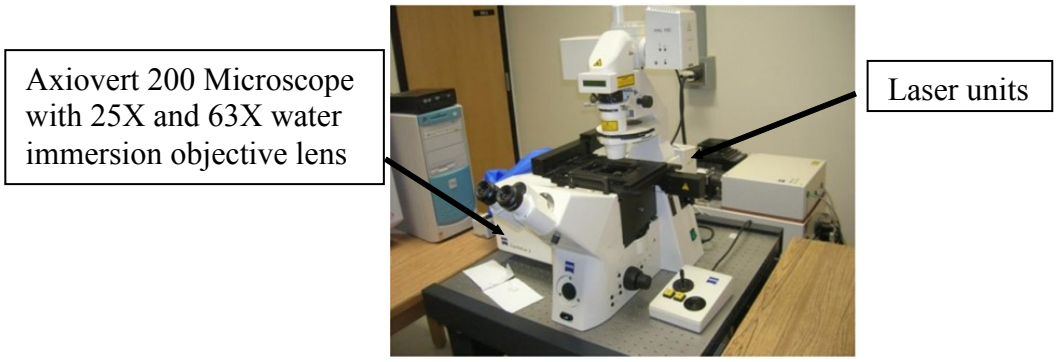
NA	+	-7.9	-10.4	dead
NA	+	-2.5	-7.4	dead
NA	+	-2.9	-7.7	dead
NA+Ca	+	-2.5	down to -23 no	dead
NA+Ca	+	-4.2	down to -23 no	dead
NA+Ca	+	-2.5	-16.0	dead
ACC	+	-5.0	one cell -17.1	alive except intracellular frozen cells
ACC	+	-2.5	down to -23 no	dead
ACC	+	-2.8	down to -23 no	dead
ACC	+	-3.2	down to -23 no	dead
ACC	+	-2.5	down to -23 no	dead

(1) NA= non-acclimation; NA+Ca= non-acclimation plus 0.05 M $CaCl_2$;
ACC= cold acclimation

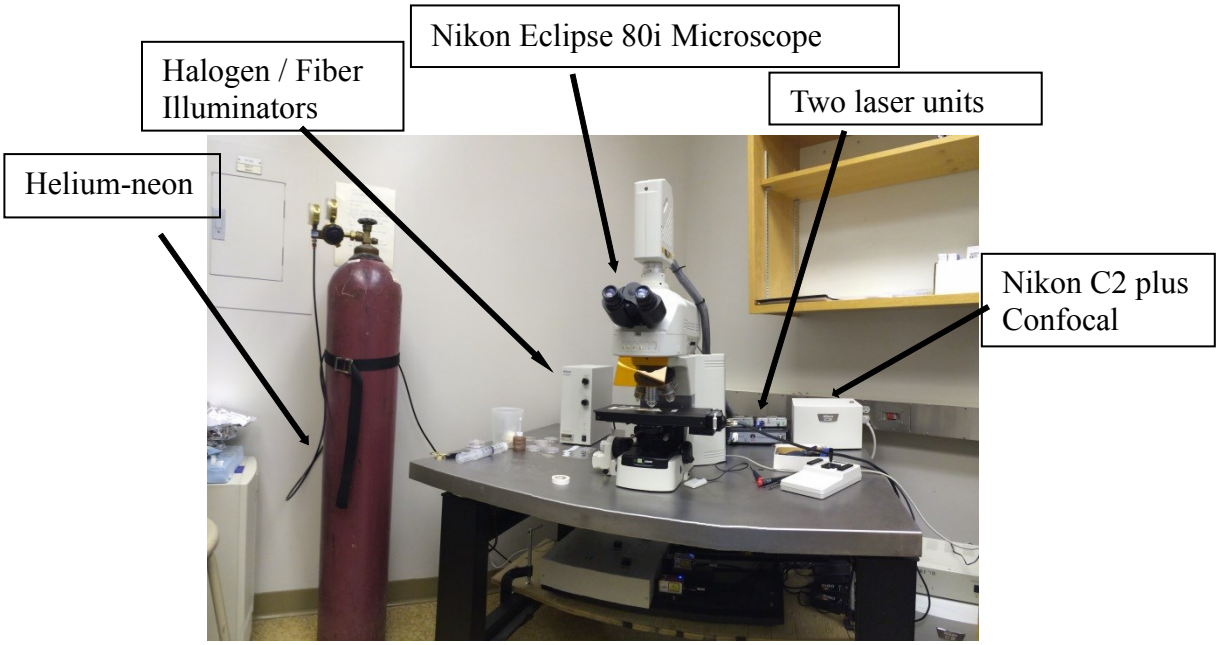
(2) +/- means added/not added extracellular nucleating agent

(3) Determined by visible protoplasmic streaming

APPENDIX E: Confocal microscope and fluorescein penetration



ZEISS LSM 510 Confor2 (Department of Biology, University of Saskatchewan)



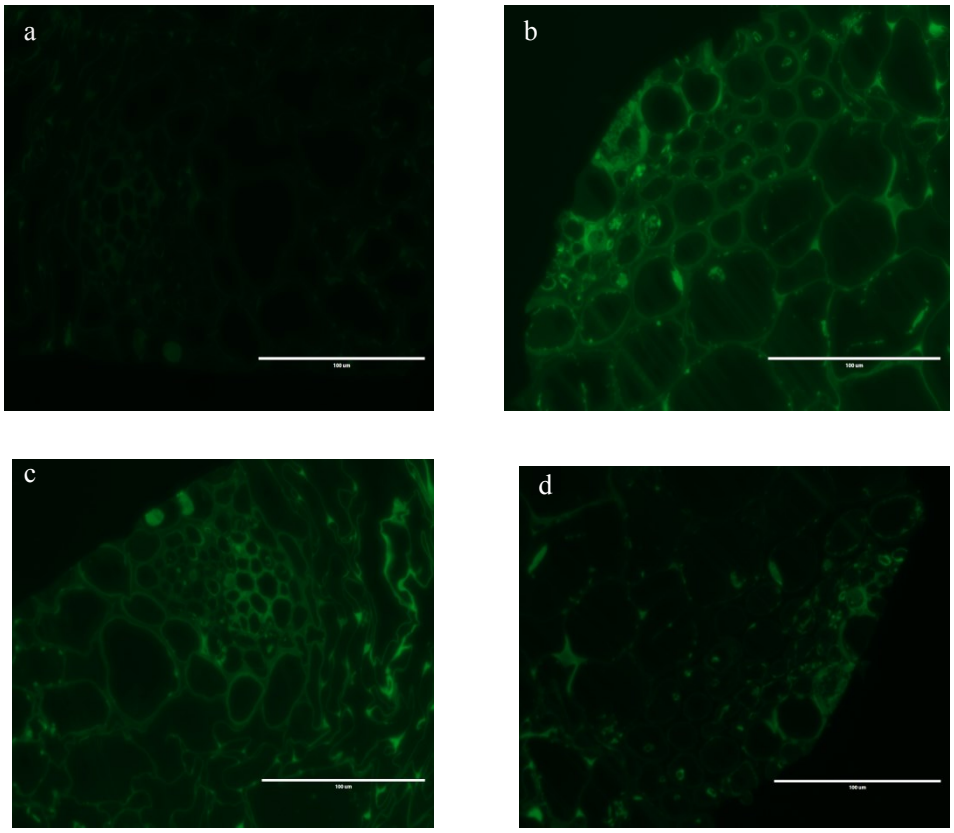
Nikon confocal microscope (National Hydrology Research Institute, Saskatoon)

media4.avi fluorescein penetration in non-acclimated cell

media5.avi fluorescein penetration in acclimated cells

APPENDIX F: Evos digital inverted microscope (Department of Plant Sciences)

APPENDIX G: Analysis of pectin methyl esterification in ligule epidermal cells



Micrographs of immunolabelling using JIM 5 and JIM 7 antibodies are shown.
a: non-acclimated sample with JIM 5; b: acclimated sample with JIM 5;
c: non-acclimated sample with JIM 7; d: acclimated sample with JIM 7.
Bars: 100 µm.